

**METHODS AND COMPOSITIONS FOR
MYCOPLASMA PNEUMONIAE EXOTOXINS**

RELATED APPLICATIONS

This application claims the benefit, under 35 U.S.C. § 119(e), of U.S. Provisional Application Serial No. 60/508,607, filed October 3, 2003, the entire contents of which are incorporated herein by reference.

STATEMENT OF GOVERNMENT SUPPORT

5 Research related to this invention was supported, at least in part, by U.S. Government Grant No. AI45737 awarded by the NIAID. The Government has certain rights in this invention.

BACKGROUND OF THE INVENTION

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FIELD OF THE INVENTION

The present invention relates to *Mycoplasma pneumoniae* exotoxins, antibodies thereto, and their use in diagnostic and therapeutic methods.

15 **BACKGROUND ART**

Mycoplasma pneumoniae is one of the most well recognized pathogens of the human respiratory tract. The importance of *Mycoplasma pneumoniae* as a cause of human respiratory disease has been well documented by epidemiological studies in various settings and in many countries. *M. pneumoniae* is the etiologic agent of 20 primary atypical pneumonia and is also responsible for many respiratory tract infections, such as tracheobronchitis, bronchiolitis, pharyngitis and croup, especially in older children and young adults and in elderly populations. It accounts for 20-30% of all pneumonias and also is linked to asthma and chronic obstructive pulmonary disease. Furthermore, *M. pneumoniae* can disseminate to other organ sites and cause 25 gastrointestinal, hematologic, neurologic, dermatologic, musculoskeletal and

cardiovascular pathologies. This secondary involvement by *M. pneumoniae* leads to a spectrum of complicated extrapulmonary sequelae, including arthritis, pericarditis and central nervous system disorders, which attests to the significance of *M. pneumoniae* in human disease. Although antibiotic therapy appears to be relatively effective in controlling mycoplasma pneumonia, the bacteria continue to persist.

At present, no known virulence determinants of *M. pneumoniae* have been functionally identified and linked to the wide range of pathologies associated with *M. pneumoniae* mediated diseases. Furthermore, there are no specific and standardized diagnostic tests available for reliable and rapid detection of *M. pneumoniae* infection, or effective vaccines to control *M. pneumoniae* infection.

The present invention overcomes previous shortcomings in the art by providing a *Mycoplasma pneumoniae* polypeptide and biologically active fragments thereof, known as community acquired respiratory distress syndrome (CARDS) toxin, as well as nucleic acids encoding this polypeptide and its fragments and antibodies specific thereto. These compositions are used, for example, in methods of diagnosing, treating and preventing infection by *M. pneumoniae*.

SOME SEQUENCES OF THIS INVENTION:

Reference amino acid sequence M129/B9 (reference strain): (SEQ ID NO:1)

MPNPVRFVYR VDLRSPEEIF EHGFSTLGDV RNFFEHILST NFGRSYFIST SETPTAAIRF
 FGSQLREYVP EHPRRAYLYE IRADQHFYNA RATGENLLDL MRQRQVVFDSDGREMAQMGI
 RALRTSFAYQ REWFTDGPIA AANVRSAWLV DAVPVEPGHA HHPAGRVTET TRINEPEMHN
 PHYQELQTQA NDQPWLPTPG IATPVHLSIP QAASVADVSE GTSASLSFAC PDWSPPSSNG
 ENPLDKCIAE KIDNYNLQSL PQYASSVKEL EDTPVYLRGKI KTQKTFMLQA DPQNNNVFLV
 EVNPKQKSSF PQTIFFWDVY QRICLKDLTG AQISLSLTAF TTQYAGQLKV HLSVSAVNAV
 NQWKWMTPQD IAITQFRVSS ELLGQTENGL FWNTKSIGGSQ HDLYVCPLKN PPSDLEELQI
 IVDECTTHAQ FVTMRAASTF FVDVQLGWYW RGYYYTPQLS GWSYQMKTQPD GQIFYDLKTS
 KIFVQDNQN VFFLHNKLNK QTGYSWDWVE WLKHDMNEDK DENFKWYFSR DDLTIPSVEG
 30 LNFRHIRCYA DNQQLKVIIS GSRWGGWYST YDKVESNVED KILVKDGFDR F

S1 (clinical strain) amino acid sequence: (SEQ ID NO:2)

MPNPVRFVYRVDLRSPEEIFEHGFSTLGDVRNFFEHIPSTNFGRSYFISTSETPTAAIRF
 FGSQLREYVP EHPRRAYLYEIRADQHFYNA RATGENLLDLMRQRQVVFDSDGREMAQMGI
 RALRTSFAYQREWFTDGPIAAANVRSAWLVDAVPVEPGHAHHPAGRVTETTRINEPEMHN
 PHYQELQTQANDQPWLPTPGIATPVHLSIPQAASVADVSEGTSASLSFACPDWSPPSSNG
 ENPLDKCIAE KIDNYNLQSLPQYASSVKELEDTPVYLRGKI KTQKTFMLQA DPQNNNVFLV
 EVNPKQKSPFPQTIFFWDVYQRICLKDLTG AQISLSLTAF TTQYAGQLKV HLSVSAVNAV
 NQWKWMTPQDSA ITQFRVSSELLGQTENGLSWNTKSIGGSQHDLYVCPLKNPPSDLEELQI
 40 IVDECTTHAQ FVTMRAASTFFFVDVQLGWYWRGYYTPQLSGWSYQMKTQPDGQIFYDLKTS

KIFFVQDNQNFFLHNKLNKQTGYSWDWVEWLKHDMNEDKDENDFKWYFSRDDLTI
PSVEGLNFRHIRCYADNQQLKVIISGSRWGGWYSTYDKVESNVEDKILVKDGFDRF

5 JL (clinical strain) amino acid sequence: (SEQ ID NO:3)

MPNPVRFVYRVDLRSPEEIFEHGFSTLGDVRNFFEHLSTNFGRSYFISTSETPTAAIRF
FGSWLREYVPEHPRRAYLYEIRADQHFYNARATGENLLDLMRQRQVVFDSGDREMAQMGI
RALRTSFAYQREWFTDGPPIAAANVRSAWLVDAPVVEPGHAHHHPAGRVVETTRINEPEMHN
PHYQELQTQANDQPWLPTPGIATPVHLSIPQAASVADVSEGTASALSACPDWSPPSSNG
ENPLDKCIAEKIDNYNLQSLPQYASSVKELEDTPVYLRLGIKTQKTFMLQADPQNNNVFLV
EVNPKQKSSFPQTIFFWDVYQRICLKDLTGAQISLSTAFTTQYAGQLKVHLSVASNAV
NQWKWMTPQDSAITQFRVSSELLGQTENGLFWNTKSGGSQHDLYVCPLKNPPSDLEELQI
IVDECTTHAQFVTMRAASTFFFDVQLGWYWRGYYYTPQLSGWSYQMKTPDGQIFYDLKTS
KIFFVQDNQNFFLHNKLNKQTGYSWDWVEWLKHDMNEDKDENDFKWYFSRDDLTI
PSVEGLNFRHIRCYADNQQLKVIISGSRWGGWYSTYDKVESNVEDKILVKDGFDRF

RJL1 (clinical strain) amino acid sequence: (SEQ ID NO:4)

20 MPNPVRFVYRVDLRSPEEIFEHGFSTLGDVRNFFEHLSTNFGRSYFISTSETPTAAIRF
FGSWLREYVPEHPRRAYLYEIRADQHFYNARATGENLLDLMRQRQVVFDSGDREMAQMGI
RALRTSFAYQREWFTDGPPIAAANVRSAWLVDAPVVEPGHAHHHPAGRVVETTRINEPEMHN
PHYQELQTQANDQPWLPTPGIATPVHLSIPQAASVADVSEGTASALSACPDWSPPSSNG
ENPLDKCIAEKIDNYNLQSLPQYASSVKELEDTPVYLRLGIKTQKTFMLQADPQNNNVFLV
EVNPKQKSSFPQTIFFWDVYQRICLKDLTGAQISLSTAFTTQYAGQLKVHLSVASNAV
NQWKWMTPQDSAITQFRVSSELLGQTENGLFRNTKSGGSQHDLYVCPLKNPPSDLEELQI
IVDECTTHAQFVTMRAASTFFFDVQLGWYWRGYYYTPQLSGWSYQMKTPDGQIFYDLKTS
KIFFVQDNQNFFLHNKLNKQTGYSWDWVEWLKHDMNEDKDENDFKWYFSRDDLTI
PSVEGLNFRHIRCYADNQQLKVIISGSRWGGWYSTYDKVESNVEDKILVKDGFDRF

30 L2 (clinical strain) amino acid sequence: (SEQ ID NO:5)

35 MPNPVRFVYRVDLRSPEEIFEHGFSTLGDVRNFFEHLSTNFGRSYFISTSETPTAAIRF
FGSWLREYVPEHPRRAYLYEIRADQHFYNARATGENLLDLMRQRQVVFDSGDREMAQMGI
RALRTSFAYQREWFTDGPPIAAANVRSAWLVDAPVVEPGHAHHHPAGRVVETTRINEPEMHN
PHYQELQTQANDQPWLPTPGIATPVHLSIPQAASVADVSEGTASALSACPDWSPPSSNG
ENPLGKCTAEKIDNYNLQSLPQYASSVKELEDTPVYLRLGIKTQKTFMLQADPQNNNVFLV
EVNPKQKSSFPQTIFFWDVYQRICLKDLTGAQISLSTAFTTQYAGQLKVHLSVASNAV
NQWKWMTPQDSAITQFRVSSELLGQTENGLFWNTKSGGSQHDLYVCPLKNPPSDLEELQI
IVDECTTHAQFVTMRAASTFFFDVQLGWYWRGYYYTPQLSGWSYQMKTPDGQIFYDLKTS
KIFFVQDNQNFFLHNKLNKQTGYSWDWVEWLKHDMNEDKDENDFKWYFSRDDLTI
PSVEGLNFRHIRCYADNQQLKVIISGSRWGGWYSTYDKVESNVEDKILVKDGFDRF

Composite amino acid sequence: (SEQ ID NO:6)

45 MPNPVRFVYR VDLRSPEEIF EHGFSTLGDV RNFFEHIPST NFGRSYFIST SETPTAAIRF
FGSWLREYVP EHPRRAYLYE IRADQHFYNA RATGENLLDL MRQRQVVFDS GDREMAQMGI
RALRTSFAYQ REWFTDGPPIA AANVRSAWLW DAVPVEPGHA HHPAGRVVET TRINEPEMHN
PHYQELQTQA NDQPWLPTPG IATPVHLSIP QAASVADVSE GTSASLSFAC PDWSPPSSNG
ENPLGKCTAE KIDNYNLQSL PQYASSVKEL EDTPVYLRLGI KTQKTFMLQA DPQNNNVFLV
EVNPKQKPSF PQTIFFWDVY QRICLKDLTG AQISLSTAFT TTQYAGQLKV HLSVASNAV
NQWKWMTPQDSAITQFRVSSELLGQTENGL SRNTKSGGSQ HDLYVCPLKN PPSDLEELQI
IVDECTTHAQ FVTMRAASTF FVDVQLGWY RGYYYTPQLS GWSYQMKTPD GQIFYDLKTS
KIFFVQDNQN VFFLHNKLNK QTGYSWDWVE WLKHDMNEDK DENFKWYFSR DDLTI
PSVEGLNFRHIRCYA DNQQLKVIIS GSRGGWYST YDKVESNVED KILVKDGFDR F

55

Reference nucleotide sequence M129/B9 (contains tga's that need to be changed to tgg before expression in *E. coli*) (SEQ ID NO:7)

5	ttttaattt	gtaaaatttc	attttttaaa	aatgccaaat	cctgttagat	ttgtttaccc
	tgttgatttg	agaagccctg	aagaaatttt	tgaacatggc	tttcaactt	taggtatgt
	gagaaaatttc	tttgaacaca	ttctctccac	taattttggg	agaagctatt	tttattccac
	ttcagaaaaca	cccacagcag	ctattcgctt	cttggtagc	tggttacggg	aatatgtacc
	agagcaccgg	agaagggttt	acttataatga	aattcgtgcc	gaccaacact	tttacaatgc
	ccgcggccact	ggggagaact	tgttagattt	aatgcgtcaa	agacaagtag	tatttactc
10	tggtgatcga	gaaatggcac	aatatggaaat	tagagctta	cgcacttcct	ttgcgtatca
	acgtgaatgg	tttaccgatg	gtccaattgc	agcagctaatt	gtccgttagt	tttgcactgt
	agatgctgtt	cccggttgaac	ctggtcatgc	tcaccacccg	gctggctgt	ttgttagagac
	tactagaattt	aatgaaccgg	aatatgcacaa	ccctcattat	caagagctgc	aaacccaagg
	caatgatcaa	ccatgattgc	caacaccagg	aatagctact	cctgtacatt	tatcaattcc
	ccaaggcagct	tccgttgcgt	atgtttcgg	aggtacttcc	gttccgctat	cgtttgcgtg
	ccctgttgg	agtccacctt	ctagtaatgg	tgaaaatccg	ctagacaaat	gcattgcggg
	aaagattgtt	aactataacc	tacaatcctt	accacagtag	gctagoagtg	taaaggaact
	ggaagatata	ccagttatcc	taaggggaaat	taaaaacgcaa	aaaaccttta	tgttacaagc
	agatccgcaa	aataacaatg	tcttttttgt	cgaagtaaac	cccaaacaaa	agtccagctt
20	tcccccaaaacc	atcttctttt	gggatgttta	tcaacgaatt	tgtctcaagg	attaactgg
	tgcacaaatc	agtcttcgc	ttaatgcctt	tactactcag	tatgctggc	agctcaaagt
	gcaccttagt	gttagcgcgg	gggtctcctc	tgaactgtta	tggaaaatga	caccgcaga
	cattgcaata	actcagttt	gtggttcaca	acacgatttg	ggtcaaactg	aaaatggctt
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	tggacagata	ttctatgatc	aactcaacaa	acaaacttgtt	tttgcagg	acaaccaaaa
	cgtgttctt	ctccataata	atgaggacaa	agacgaaaac	tacagctggg	attgagtaga
25	atggctaaaa	catgacatga	ccgttgaagg	gcttaacttc	tttaaatgg	acttttcgcg
	tgatgacctt	accattcctt	tgatcataag	cggttcacgt	cgccacattc	gctgttacgc
	tgacaaccag	cagttaaagg	atgtcgaaga	taagattttg	tggggcggtt	ggtaactccac
	ttacgataaa	gttggaaagta	acgtcaactgt	tttgccttaa	gtcaaagatg	gttttgcacg
	cttttagcga	ttaagcttta			tgttagaaagc	aaagatcttg
30						
35						

S1 Nucleotide sequence with each tga changed to tgg for expression in *E. coli* (SEQ ID NO:8)

40	atgccaaatc	ctgttagatt	tgttaccgt	gttgatttga	gaagccctga	agaaaatttt	60
	gaacatggct	tttcaacttt	aggtgatgtg	agaaaatttct	ttgaacacat	tccctccact	120
	aattttggta	gaagcttattt	tatttccact	tcaaaaaacac	ccacagcgc	tattcgcttc	180
	tttggtagct	ggttacggga	atatgttacca	gagccccca	gaagggttta	tttatatatgaa	240
	attcgtgcgg	accacacactt	ttacaatgcc	cgcgcactg	gggagaactt	tttagatttta	300
45	atgcgtcaaa	gacaagtgt	atttgactct	ggtgatcgag	aatggcaca	aatgggaaatt	360
	agagctttac	gcacttcctt	tgcgtatcaa	cgtgaatgggt	ttaccgttgg	tccaatttgc	420
	gcagctaattg	tccgtatgtc	ttggctatgt	gatgttgc	ccgttgaaacc	ttgtcatgt	480
	caccacccgg	ctggtcgtgt	tgttagagact	actagaatttta	atgaaccggaa	aatgcacaac	540
	cctcattatc	aagagctgca	aacccaaagcc	aatgtatcaac	catgggttgc	aacaccaggaa	600
50	atagctactc	ctgtacattt	atcaattccc	caagcagctt	ccgttgctga	tttttcggaa	660
	ggtacttccg	cttcgcgtatc	gtttgcgtgc	cctgatttggaa	gtccacccctt	tagtaatgtt	720
	aaaaatccgc	tagacaaaatg	cattgcggaa	aagattgtata	actataaacct	acaatccctt	780
	ccacagtcg	ctagcgttgt	aaaggaactg	gaagatacac	cagtataacct	aaggggaaatt	840
	aaaacgc当地	aaacctttat	gttacaagca	gatccgc当地	ataacaatgt	ctttttggc	900
55	gaagtaaacc	ccaaacaaaa	gccccagctt	ccccaaacca	tcttcttttgc	ggatgtttat	960
	caacgaattt	gtctcaagga	tttaactgggt	gcacaaaatca	gtctttcgct	tactgccttt	1020
	actactcagt	atgctgggtca	gctcaaagt	caccttagtgc	ttagcgc当地	taatgccgtg	1080
	aacccaaaatg	ggaaaatgac	accgcaagac	agtgcaataa	ctcagttcg	ggtctccctt	1140
	gaactgttag	gtcaaactga	aaatggcttgc	tcctqqaata	ccaaqagttqq	ttqgttcacaa	1200

	cacgatttgt atgtatgtcc tttggaaaaat ccaccttagt gttgatgttga aatccaaata 1260
	atgttgatg aatgtactac ccatgcgcag ttgttacta tgcgtgcagc tagcaccttc 1320
	ttgttgatg ttcagctagg ctggtagtgg aggggttatt actatacccc acaattaagt 1380
5	ggttggctt atcagatgaa aacaccagat ggacagatat tctatgatct aaaaacttcg 1440
	aaaatctctt ttgtccagga caacccaaac gtgttcttc tccataataa actcaacaaa 1500
	caaactgggtt acagctggga ttgggttagaa tggctaaaac atgacatgaa tgaggacaaa 1560
	gacgaaaact ttaaatggta cttttcgct gatgacctt ccattccttc cggtgaaggg 1620
10	cttaacttcc gccacattcg ctgttacgt gacaaccagc agttaaaggt gatcataagc 1680
	ggttacgtt gggcggtt gtaactccact tacgataaaag ttgaaagtaa tgtcgaagat 1740
	aagattttgg tcaaagatgg ttttgcgc ttt 1773

L2 nucleotide sequence with each tga changed to tgg for expression in *E. coli* (SEQ ID NO:9)

15	atgccaaatc ctgttagatt tgtttaccgt gttgattga gaagccctga agaaattttt 60
	gaacatggct tttcaactt aggtgatgtg agaaatttct ttgaacacat tctctccact 120
	aattttggta gaagctattt tatttccact tcagaaaacac ccacagcagc tattcgcttc 180
	tttggtagct ggttacggga atatgtacca gaggcaccctt gaagggttta cttatatgaa 240
20	attcgtgccg accaacactt ttacaatgcc cgcccaactg gggagaactt gttagattt 300
	atgcgtcaaa gacaagtagt atttgactct ggtgatcgag aaatggcaca aatgggatt 360
	agagcttac gcacttccct tgcgtatcaa cgtgaatggt ttaccgtatgg tccaattgca 420
	gcagctaattg tccgttagtgc ttggcttagt gatgtgttc ccgttgaacc tggcatgct 480
	caccacccgg ctggtcgtgt ttttagagact actagaatta atgaaaccggaa aatgcacaac 540
25	cctcattatc aagagctgca aacccaaagcc aatgtacaac catggttgcc aacaccagga 600
	atagctactc ctgtacattt atcaattccc caagcagctt ccgttgcgtga tgtttggaa 660
	ggtacttccg cttcgcatac gtttgcgtgc cctgatttggaa gtccacccctt tagaatgg 720
	gaaaatccgc taggcaatgt cattgcggaa aagattgata actataaccc acaatccctt 780
	ccacagtagc cttagcgtgt aaaggaactg gaagatacac cagtataacct aaggggatt 840
30	aaaacgcaaa aaacccattt gttacaagca gatccgc当地 aatacaatgt ctttttggc 900
	gaagtaaacc ccaaacaaaaa gtcgc当地 ccccaaaccat tcttcttttggatgtttat 960
	caacgaattt gtctcaagga tttaacttggt gcacaaaatca gtcttcgc当地 tactgcctt 1020
	actacttagt atgctgtca gtc当地 aatggcgtt cacccttagt gtagcgc当地 taatgc当地 1080
	aaccaaaaatg gaaaaatgac accgcaagac agtgc当地 aatggcgtt ctc当地 aatggcgtt 1140
35	gaactgttagt gtc当地 aatggcgtt ttcttggaa ccaagagtttgg tggccaca 1200
	cacgatttgt atgtatgtcc ttggaaaaat ccacccatgtt gtttggaaata attacaatata 1260
	atgttgatg aatgtactac ccatgcgc当地 ttgttacta tgcgtgc当地 tagcaccttc 1320
	tttggtagt ttcagctagg ctggtagtgg aggggttatt actatacccc acaattaagt 1380
	ggttggctt atcagatgaa aacaccagat ggacagatat tctatgatct aaaaacttcg 1440
40	aaaatctctt ttgtccagga caacccaaac gtgttcttc tccataataa actcaacaaa 1500
	caaactgggtt acagctggga ttgggttagaa tggctaaaac atgacatgaa tgaggacaaa 1560
	gacgaaaact ttaaatggta cttttcgct gatgacctt ccattccttc cggtgaaggg 1620
	cttaacttcc gccacattcg ctgttacgt gacaaccagc agttaaaggt gatcataagc 1680
	ggttacgtt gggcggtt gtaactccact tacgataaaag ttgaaagtaa tgtcgaagat 1740
45	aagattttgg tcaaagatgg ttttgcgc ttt 1773

JL nucleotide sequence with each tga changed to tgg for expression in *E. coli* (SEQ ID NO:10)

50	atgccaaatc ctgttagatt tgtttaccgt gttgattga gaagccctga agaaattttt 60
	gaacatggct tttcaactt aggtgatgtg agaaatttct ttgaacacat tctctccact 120
	aattttggta gaagctattt tatttccact tcagaaaacac ccacagcagc tattcgcttc 180
	tttggtagct ggttacggga atatgtacca gaggcaccctt gaagggttta cttatatgaa 240
	attcgtgccg accaacactt ttacaatgcc cgcccaactg gggagaactt gttagattt 300
55	atgcgtcaaa gacaagtagt atttgactct ggtgatcgag aaatggcaca aatgggatt 360
	agagcttac gcacttccct tgcgtatcaa cgtgaatggt ttaccgtatgg tccaattgca 420
	gcagctaattg tccgttagtgc ttggcttagt gatgtgttc ccgttgaacc tggcatgct 480
	caccacccgg ctggtcgtgt ttttagagact actagaatta atgaaaccggaa aatgcacaac 540
	cctcattatc aagagctgca aacccaaagcc aatgtacaac catggttgcc aacaccagga 600
	atagctactc ctgtacattt atcaattccc caagcagctt ccgttgcgtga tgtttggaa 660

	ggtaacttccg	cttcgcatac	gtttgcgtgc	cctgatttga	gtccaccccttc	tagtaatgg	720
	aaaaatccgc	tagacaatg	cattgcggaa	aagattgata	actataacct	acaatcctta	780
	ccacagtacg	ctagcatgt	aaaggaactg	gaagatacac	cagtatact	aaggggatt	840
5	aaaacgcaaa	aaaccttat	gttacaagca	gatccgc当地	ataacaatgt	cttttgg	900
	gaagtaaaccc	ccaaacaaaa	gtccagctt	ccccaaacca	tcttctttt	ggatgtttat	960
	caacgaattt	gtctcaagga	ttaactgg	gcacaaatca	gtcttcgct	tactgcctt	1020
	actactcagt	atgctgg	gtcaaaatgt	caccttagt	ttagcgc当地	taatgccgt	1080
10	aaccaaaaat	ggaaaatgac	accgcaagac	agtgc当地aa	ctcagttcg	ggtctcc	1140
	gaactgttag	gtcaaactga	aatggctt	ttctggaata	ccaagagtg	tgg	1200
	cacgatttgt	atgtatgtcc	tttggaaaat	ccaccttagt	atttggaaaga	attacaata	1260
	atttgtatg	aatgtactac	ccatgc当地	tttggtaact	tgcgtgc当地	tagcacctc	1320
	tttgtatg	ttcagctagg	ctggtagtgg	aggggttatt	actataccc	acaattaagt	1380
	ggttgtctt	atcagatgaa	aacaccagat	ggacagat	tctatgatct	aaaaacttcg	1440
15	aaaatcttct	ttgtccagga	caacccaaac	gtgttctt	tccataataa	actcaacaa	1500
	caaactgg	acagctgg	ttgggttagaa	tggctaaac	atgacatgaa	tgaggacaa	1560
	gacaaaact	ttaaatggta	ctttcgc当地	gatgaccta	ccattcctc	cgttgaagg	1620
	cttaacttcc	gccacattcg	ctgttacg	gacaaccagc	agttaaaggt	gatcataagc	1680
	ggtcacgtt	ggggcggtt	gtactccact	tacgataaaag	ttgaaagtaa	tgtcgaagat	1740
20	aagatttgg	tcaaagatgg	ttttgatcgc	ttt			1773

RJL1 nucleotide sequence with each tga changed to tgg for expression in *E. coli* (SEQ ID NO:11)

25	atgccaaatc	ctgttagatt	tgtttaccgt	gttgatttga	gaagccctga	agaaatttt	60
	gaacatggct	tttcaactt	aggtgatgt	agaaatttt	ttgaacacat	tctctccact	120
	aattttggta	gaagctattt	tatttccact	tcagaaacac	ccacagcagc	tattcgctt	180
	tttggtagct	gttacgg	atatgtacca	gagcacc	gaagggctt	tttatatgaa	240
	attcg	accacactt	ttacaatg	cgc当地	gggagaactt	gttagattt	300
30.	atgcgtcaaa	gacaagtagt	atttgc	gtgtatcg	aatggcaca	aatggaaatt	360
	agagcttac	gcacttcc	tgc当地	cgtgaatgg	ttaccgatgg	tccaaattgca	420
	gcagcta	tccgtatg	ttggctag	gatgctgtt	ccgttgaacc	tggcatg	480
	caccacccgg	ctggctgt	tgtagagact	actagaatta	atgacccg	aatgcacaac	540
	cctcattatc	aagagctg	aa	aatgatcaac	catgg	aacaccag	600
35	atagctact	ctgtacattt	atcaatttcc	caagcagctt	ccgttgc当地	tgttccgg	660
	ggtacttccg	cttgc当地	gttgc当地	cctgatttga	gtccaccc	tagtaatgg	720
	aaaaatccg	tagacaatg	cattgc当地	aagattgata	actataacct	acaatcctt	780
	ccacagtacg	ctagcatgt	aaaggaactg	gaagatacac	cagtatact	aaggggatt	840
	aaaacgcaaa	aaaccttat	gttacaagca	gatccgc当地	ataacaatgt	cttttgg	900
40	gaagtaaaccc	ccaaacaaaa	gtccagctt	ccccaaacca	tcttctttt	ggatgtttat	960
	caacgaattt	gtctcaagga	ttaactgg	gcacaaatca	gtcttcgct	tactgcctt	1020
	actactcagt	atgctgg	gtcaaaatgt	caccttagt	ttagcgc当地	taatgccgt	1080
	aaccaaaaat	ggaaaatgac	accgcaagac	agtgc当地aa	ctcagttcg	ggtctcc	1140
	gaactgttag	gtcaaactga	aatggctt	ttceggaata	ccaagagtg	tgg	1200
	cacgatttgg	atgtatgtcc	tttggaaaat	ccaccttagt	atttggaaaga	attacaata	1260
45	atttgtatg	aatgtactac	ccatgc当地	tttggtaact	tgcgtgc当地	tagcacctc	1320
	tttgtatg	ttcagctagg	ctggtagtgg	aggggttatt	actataccc	acaattaagt	1380
	ggttgtctt	atcagatgaa	aacaccagat	ggacagat	tctatgatct	aaaaacttcg	1440
	aaaatcttct	ttgtccagga	caacccaaac	gtgttctt	tccataataa	actcaacaa	1500
50	caaactgg	acagctgg	ttgggttagaa	tggctaaac	atgacatgaa	tgaggacaa	1560
	gacaaaact	ttaaatggta	ctttcgc当地	gatgaccta	ccattcctc	cgttgaagg	1620
	cttaacttcc	gccacattcg	ctgttacg	gacaaccagc	agttaaaggt	gatcataagc	1680
	ggtcacgtt	ggggcggtt	gtactccact	tacgataaaag	ttgaaagtaa	tgtcgaagat	1740
	aagatttgg	tcaaagatgg	ttttgatcgc	ttt			1773

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows an immunoblot that demonstrates both production of the
5 CARDS toxin and anti-CARDS antibodies in three patients during infection with
Mycoplasma pneumoniae.

Figure 2 shows ADP-ribosylation of G proteins in HEp-2 cells following
incubation with CARDS protein. Lane 1: HEp-2 cells in medium alone followed by
preparation of cell free extract and addition of CARDS protein. Lane 2: HEp-2 cells
10 pretreated with CARDS protein, followed by preparation of cell free extract and
addition of CARDS protein. The marked reduction in ADP-ribosylation of specific
proteins in the CARDS protein-pretreated cells is indicated by arrows. Also, ADP-
ribosylation of other Hep-2 cell proteins is diminished (lane 2).

Figure 3 shows an ELISA and an immunoblot employing rD1 as antigen that
15 demonstrates production of anti-CARDS antibodies in sequential serum samples of two
patients infected with *Mycoplasma pneumoniae*.

SUMMARY OF THE INVENTION

The present invention provides *Mycoplasma pneumoniae* exotoxin (CARDS
20 toxin) from subjects infected with *Mycoplasma pneumoniae*. In particular, the present
invention provides a polypeptide comprising, consisting essentially of, and/or
consisting of the amino acid sequence of SEQ ID NO:2 (S1 isolate), a polypeptide
comprising, consisting essentially of, and/or consisting of the amino acid sequence of
SEQ ID NO:3 (JL isolate), a polypeptide comprising, consisting essentially of, and/or
25 consisting of the amino acid sequence of SEQ ID NO:4 (RJL1 isolate), a polypeptide
comprising, consisting essentially of, and/or consisting of the amino acid sequence of
SEQ ID NO:5(L2 isolate), a polypeptide comprising, consisting essentially of, and/or
consisting of the amino acid sequence of SEQ ID NO:1(reference sequence), and/or a
polypeptide comprising, consisting essentially of, and/or consisting of the amino acid

sequence of SEQ ID NO:6 (composite sequence), either individually or in any combination.

The present invention further provides biologically active fragments of the polypeptides of this invention, as well as antibodies that specifically bind the
5 polypeptides and/or fragments of the polypeptides of this invention.

Further provided are nucleotide sequences that encode the polypeptides and fragments of this invention. In particular, the present invention provides an isolated nucleic acid comprising, consisting essentially of, and/or consisting of the nucleotide sequence of SEQ ID NO:8 (S1 isolate), an isolated nucleic acid comprising, consisting essentially of, and/or consisting of the nucleotide sequence of SEQ ID NO:10 (JL
10 isolate), an isolated nucleic acid comprising, consisting essentially of, and/or consisting of the nucleotide sequence of SEQ ID NO:11 (RJL1 isolate), an isolated nucleic acid comprising, consisting essentially of, and/or consisting of the nucleotides sequence of SEQ ID NO:9 (L2 isolate), an isolated nucleic acid comprising, consisting essentially of, and/or consisting of the nucleotides sequence of SEQ ID NO:7 (reference sequence),
15 and/or an isolated nucleic acid comprising, consisting essentially of, and/or consisting of the nucleotide sequence of SEQ ID NO:76 (composite sequence), either individually or in any combination.

Additionally provided is a nucleic acid comprising, consisting essentially of,
20 and/or consisting of a nucleotide sequence that encodes an amino acid sequence comprising, consisting essentially of, and/or consisting of the amino acid sequence or a biologically active fragment of the amino acid sequence of SEQ ID NO:2 (S1 isolate), a nucleic acid comprising, consisting essentially of, and/or consisting of a nucleotide sequence that encodes an amino acid sequence comprising, consisting essentially of,
25 and/or consisting of the amino acid sequence or a biologically active fragment of the amino acid sequence of SEQ ID NO:3 (JL isolate), a nucleic acid comprising, consisting essentially of, and/or consisting of a nucleotide sequence that encodes an amino acid sequence comprising, consisting essentially of, and/or consisting of the amino acid sequence or a biologically active fragment of the amino acid sequence of
30 SEQ ID NO:4 (RJL1 isolate), a nucleic acid comprising, consisting essentially of,

and/or consisting of a nucleotide sequence that encodes an amino acid comprising, consisting essentially of, and/or consisting of the amino acid sequence or a biologically active fragment of an amino acid sequence of SEQ ID NO:5 (L2) isolate, a nucleic acid comprising, consisting essentially of, and/or consisting of a nucleotide sequence that
5 encodes an amino acid comprising, consisting essentially of, and/or consisting of the amino acid sequence or a biologically active fragment of an amino acid sequence of SEQ ID NO:1 (reference sequence) isolate, and/or a nucleic acid comprising, consisting essentially of, and/or consisting of a nucleotide sequence encoding an amino acid sequence comprising, consisting essentially of, and/or consisting of the amino acid
10 sequence or a biologically active fragment of the amino acid sequence of SEQ ID NO:6 (composite sequence). Further provided herein is a nucleic acid that is the complement of each and any of the nucleic acids of this invention.

Also provided herein are probes and primers for the detection and/or amplification of the nucleic acids of this invention, including
15 TTTTACATATGCCAAATCCTGTT (SEQ ID NO:12; Primer 1),
CGTTAAAGGATCCTCGCTAAAAGCGATC (SEQ ID NO:13; Primer 2),
CTAGCCAAGCACTACGGACATTAGC (SEQ ID NO:14; (Primer 3),
CGTAGTGCTTGGCTAGTAGATGCTGTT (SEQ ID NO:15; (Primer 4),
CCTGGTGTGGCAACCATGGTTG (SEQ ID NO:16; (Primer 5),
20 GATCAACCATGGTGCCAACACC (SEQ ID NO:17; (Primer 6),
AAGGTGGACTCCAATCAGGGCACG (SEQ ID NO:18; (Primer 7),
CGTGCCTGATTGGAGTCCACCTT (SEQ ID NO:19; (Primer 8),
GCGGTGTCATTTCCACTTTGG (SEQ ID NO:20; (Primer 9),
CCAAAAGTGGAAAATGACACCGC (SEQ ID NO:21; (Primer 10),
25 GGTATTCCAGAACAGCCATT (SEQ ID NO:22; (Primer 11),
GCTTGTCTGGAATACCAAGAGTG (SEQ ID NO:23; (Primer 12),
ATAACCCCTATACCAGCCTAG (SEQ ID NO:24; (Primer 13),
GCTGGTATTGGAGGGTTATTACTATACCCCACAATTAAAGTGGTTGGTCTTA
TCAGATG (SEQ ID NO:25; (Primer 14), CCATTCTACCCAATCCCAGCTGTA

(SEQ ID NO:26; (Primer 15), and TACAGCTGGATTGGGTAGAATGG (SEQ ID NO:27; (Primer 16).

Additionally provided in this invention are methods of diagnosing infection by *M. pneumoniae* in a subject comprising contacting a biological sample from the 5 subject with a polypeptide or antibody of this invention under conditions whereby an antigen/antibody complex can form; and detecting formation of an antigen/antibody complex, thereby diagnosing infection by *M. pneumoniae* in the subject.

Methods are also provided herein for diagnosing infection by *M. pneumoniae* in a subject comprising contacting a biological sample from the subject with a nucleic acid 10 of this invention under conditions whereby hybridization of nucleic acid molecules can occur; and detecting hybridization, thereby diagnosing infection by *M. pneumoniae* in the subject.

Furthermore, the present invention provides methods of eliciting an immune response in a subject, comprising administering to the subject an effective amount of a 15 polypeptide and/or biologically active fragment of a polypeptide of this invention and/or by administering to a subject an effective amount of a nucleic acid comprising a nucleotide sequence encoding a polypeptide and/or biologically active fragment of a polypeptide of this invention.

The present invention additionally provides methods of providing passive 20 immunity to a subject, comprising administering to the subject an effective amount of an antibody of this invention.

In further embodiments, the present invention provides methods of treating and/or preventing infection by *M. pneumoniae* in a subject, comprising administering to the subject an effective amount of a polypeptide of this invention and/or an effective 25 amount of a biologically active fragment of a polypeptide of this invention and/or an effective amount of a nucleic acid comprising a nucleotide sequence encoding a polypeptide of this invention and/or an effective amount of a nucleic acid comprising a nucleotide sequence encoding a biologically active fragment of a polypeptide of this invention. Also provided are methods of treating and/or preventing infection by *M.*

pneumoniae in a subject, comprising administering to the subject an effective amount of an antibody of this invention.

In yet further embodiments, the present invention provides methods of identifying substances having the ability to inhibit or enhance various activities of the 5 polypeptides and/or biologically active fragments of this invention, including but not limited to, binding activity, translocating activity, immunogenic activity, ADP-ribosylating activity and/or cytopathology inducing activity. These methods are carried out by contacting the polypeptides and/or biologically active fragments of this invention and/or the nucleic acids of this invention, with the substance to be tested for inhibitory 10 or enhancing activity, under conditions whereby the inhibition or enhancement of activity can be detected, as described herein.

Various other objectives and advantages of the present invention will become apparent from the following detailed description.

15

DETAILED DESCRIPTION OF THE INVENTION

As used herein, "a," "an" or "the" can mean one or more than one. For example, "a" cell can mean a single cell or a multiplicity of cells.

The present invention is based on the discovery of polypeptides of *Mycoplasma pneumoniae* having the respective amino acid sequence described herein and encoded 20 by the nucleic acids described herein and the identification of activities of these polypeptides and various fragments or "domains" of these polypeptides. Characterization of these polypeptides and fragments indicates that the newly identified protein is an exotoxin of *Mycoplasma pneumoniae* and it is referred to herein as 25 community acquired respiratory distress syndrome (CARDS) toxin. Thus, the present invention provides an isolated polypeptide comprising, consisting essentially of, and/or consisting of the amino acid sequence of SEQ ID NO:2 (S1 isolate), an isolated polypeptide comprising, consisting essentially of, and/or consisting of the amino acid sequence of SEQ ID NO:3 (JL isolate), an isolated polypeptide comprising, consisting essentially of, and/or consisting of the amino acid sequence of SEQ ID NO:4 (RJL1 30 isolate), an isolated polypeptide comprising, consisting essentially of, and/or consisting

of the amino acid sequence of SEQ ID NO:5 (L2 isolate), an isolated polypeptide comprising, consisting essentially of, and/or consisting of the amino acid sequence of SEQ ID NO:1 (reference sequence), and/or an isolated polypeptide comprising, consisting essentially of, and/or consisting of the amino acid sequence of SEQ ID NO:6
5 (composite sequence), either individually or in any combination.

The present invention further provides biologically active fragments of the polypeptides of this invention, as well as antibodies that specifically bind the polypeptides and/or fragments of the polypeptides of this invention.

Further provided are nucleotide sequences that encode the polypeptides and
10 fragments of this invention. In particular, the present invention provides an isolated nucleic acid comprising, consisting essentially of, and/or consisting of the nucleotide sequence of SEQ ID NO:8 (S1 isolate), an isolated nucleic acid comprising, consisting essentially of, and/or consisting of the nucleotide sequence of SEQ ID NO:10 (JL isolate), an isolated nucleic acid comprising, consisting essentially of, and/or consisting
15 of the nucleotide sequence of SEQ ID NO:11 (RJL1 isolate), an isolated nucleic acid comprising, consisting essentially of, and/or consisting of the nucleotides sequence of SEQ ID NO:9 (L2 isolate), an isolated nucleic acid comprising, consisting essentially of, and/or consisting of the nucleotides sequence of SEQ ID NO:7 (reference sequence), and/or an isolated nucleic acid comprising, consisting essentially of, and/or consisting
20 of the nucleotide sequence of SEQ ID NO:76 (composite sequence), either individually or in any combination.

Additionally provided is a nucleic acid comprising, consisting essentially of, and/or consisting of a nucleotide sequence that encodes an amino acid sequence comprising, consisting essentially of, and/or consisting of the amino acid sequence or a
25 biologically active fragment of the amino acid sequence of SEQ ID NO:2 (S1 isolate), a nucleic acid comprising, consisting essentially of, and/or consisting of a nucleotide sequence that encodes an amino acid sequence comprising, consisting essentially of, and/or consisting of the amino acid sequence or a biologically active fragment of the amino acid sequence of SEQ ID NO:3 (JL isolate), a nucleic acid comprising,
30 consisting essentially of, and/or consisting of a nucleotide sequence that encodes an

amino acid sequence comprising, consisting essentially of, and/or consisting of the amino acid sequence or a biologically active fragment of the amino acid sequence of SEQ ID NO:4 (RJL1 isolate), a nucleic acid comprising, consisting essentially of, and/or consisting of a nucleotides sequence that encodes an amino acid comprising,

5 consisting essentially of, and/or consisting of the amino acid sequence or a biologically active fragment of an amino acid sequence of SEQ ID NO:5 (L2 isolate), and/or a nucleic acid comprising, consisting essentially of, a nucleic acid comprising, consisting essentially of, and/or consisting of a nucleotides sequence that encodes an amino acid comprising, consisting essentially of, and/or consisting of the amino acid sequence or a

10 biologically active fragment of an amino acid sequence of SEQ ID NO:1 (reference sequence), and/or a nucleic acid comprising, consisting essentially of, and/or consisting of a nucleotide sequence encoding an amino acid sequence comprising, consisting essentially of, and/or consisting of the amino acid sequence or a biologically active fragment of the amino acid sequence of SEQ ID NO:6 (composite sequence). Further

15 provided herein is a nucleic acid that is the complement of each and any of the nucleic acids of this invention.

Also provided herein are probes and primers for the detection of the nucleic acids of this invention, including TTTTACATATGCCAAATCCTGTT (SEQ ID NO:12; Primer 1), CGTTAAAGGATCCTCGCTAAAAGCGATC (SEQ ID NO:13; Primer 2), CTAGCCAAGCACTACGGACATTAGC (SEQ ID NO:14; (Primer 3), CGTAGTGCTTGGCTAGTAGATGCTGTT (SEQ ID NO:15; (Primer 4), CCTGGTGTGGCAACCATGGTT (SEQ ID NO:16; (Primer 5), GATCAACCATGGTTGCCAACACC (SEQ ID NO:17; (Primer 6), AAGGTGGACTCCAATCAGGGCACG (SEQ ID NO:18; (Primer 7),

20 CGTGCCTGATTGGAGTCCACCTT (SEQ ID NO:19; (Primer 8), GCGGTGTCATTTCCACTTTGG (SEQ ID NO:20; (Primer 9), CAAAAAGTGGAAAATGACACCGC (SEQ ID NO:21; (Primer 10), GGTATTCCAGAACAGCCATT (SEQ ID NO:22; (Primer 11), GCTTGTCTGGAATACCAAGAGTG (SEQ ID NO:23; (Primer 12),

25 ATAACCCCTATAACCAGCCTAG (SEQ ID NO:24; (Primer 13),

GCTGGTATTGGAGGGGTTATTACTATACCCACAATTAAGTGGTTGGTCTTA
TCAGATG (SEQ ID NO:25; (Primer 14), CCATTCTACCCAATCCCAGCTGTA
(SEQ ID NO:26; (Primer 15), and TACAGCTGGATTGGGTAGAATGG (SEQ ID
NO:27; (Primer 16), alone and/or in any combination. The present invention further
5 provides as additional embodiments without limitation, other oligonucleotides listed in
this application and in the Sequence Listing attached hereto.

“Isolated” as used herein means the nucleic acid or polypeptide of this invention
is sufficiently free of contaminants or cell components with which nucleic acids or
polypeptides normally occur. “Isolated” does not mean that the preparation is
10 technically pure (homogeneous), but it is sufficiently pure to provide the nucleic acid or
polypeptide in a form in which it can be used therapeutically.

“Epitope” or “antigenic epitope” or “antigenic peptide” as used herein means a
specific amino acid sequence of limited length which, when present in the proper
conformation, provides a reactive site for an antibody or T cell receptor. The
15 identification of epitopes on antigens can be carried out by immunology protocols that
are well known in the art.

As used herein, the term “polypeptide” or “protein” is used to describe a chain
of amino acids that correspond to those encoded by a nucleic acid. A polypeptide of
this invention can be a peptide, which usually describes a chain of amino acids of from
20 two to about 30 amino acids. The term polypeptide as used herein also describes a
chain of amino acids having more than 30 amino acids and can be a fragment or
domain of a protein or a full length protein. Furthermore, as used herein, the term
polypeptide can refer to a linear chain of amino acids or it can refer to a chain of amino
acids that has been processed and folded into a functional protein. It is understood,
25 however, that 30 is an arbitrary number with regard to distinguishing peptides and
polypeptides and the terms can be used interchangeably for a chain of amino acids. The
polypeptides of the present invention are obtained by isolation and purification of the
polypeptides from cells where they are produced naturally, by enzymatic (e.g.,
proteolytic) cleavage, and/or recombinantly by expression of nucleic acid encoding the
30 polypeptides or fragments of this invention. The polypeptides and/or fragments of this

invention can also be obtained by chemical synthesis or other known protocols for producing polypeptides and fragments.

The amino acid sequences disclosed herein are presented in the amino to carboxy direction, from left to right. Nucleotide sequences are presented herein by single strand 5 only, in the 5' to 3' direction, from left to right. However, it is intended that the nucleic acids of this invention can be either single or double stranded (i.e., including the complementary nucleic acid). A nucleic acid of this invention can be the complement of a nucleic acid described herein.

A "biologically active fragment" includes a polypeptide of this invention that 10 comprises a sufficient number of amino acids to have one or more of the biological activities of the polypeptides of this invention. Such biological activities can include, but are not limited to, in any combination, binding activity, translocating activity, immunogenic activity, ADP-ribosylating activity, and/or cytopathology inducing activity, as well as any other activity now known or later identified for the polypeptides 15 and/or fragments of this invention. A fragment of a polypeptide of this invention can be produced by methods well known and routine in the art. Fragments of this invention can be produced, for example, by enzymatic or other cleavage of naturally occurring peptides or polypeptides or by synthetic protocols that are well known. Such fragments can be tested for one or more of the biological activities of this invention according to 20 the methods described herein, which are routine methods for testing activities of polypeptides, and/or according to any art-known and routine methods for identifying such activities. Such production and testing to identify biologically active fragments of the polypeptides described herein would be well within the scope of one of ordinary skill in the art and would be routine.

25 Fragments of the polypeptides of this invention are preferably at least about ten amino acids in length and retain one or more of the biological activities and/or the immunological activities of the CARDs toxin. Examples of the fragments of this invention include, but are not intended to be limited to, the following fragments identified by the amino acid number as shown in the Sequence Listing for each of the 30 isolates of SEQ ID NO:2 (SI isolate), SEQ ID NO:3 (JL isolate), SEQ ID NO:4 (RJL1

isolate), SEQ ID NO:5 (L2 isolate), SEQ ID NO:6 (composite sequence) and SEQ ID NO:1 (reference sequence): Amino acids 1-10, 10-20, 20-30, 30-40, 40-50, 50-60, 60-70, 70-80, 80-90, 90-100, 110-120, 120-130, 130-140, 140-150, 150-160, 160-170, 170-180, 180-190, 190-200, 200-210, 210-220, 220-230, 230-240, 240-250, 250-260, 5 260-270, 270-280, 280-290, 290-300, 300-310, 310-320, 320-330, 330-340, 340-350, 350-360, 360-370, 370-380, 380-390, 390-400, 400-410, 410-420, 420-430, 430-440, 440-450, 450-460, 460-470, 470-480, 480-490, 490-500, 500-510, 510-520, 520-530, 530-540, 540-550, 550-560, 560-570, 570-580, 580-591, 1-25, 1-50, 1-67, 1-75, 1-100, 10 1-125, 1-135, 1-145, 1-150, 1-160, 1-170, 1-180, 1-190, 1-200, 1-250, 1-300, 1-350, 1-400, 1-450, 1-500, 68-180, 183-123, 500-591, 450-591, 400-591, 350-591, 300-591, 250-591, 200-591, 150-591, 100-591, 50-591, 50-100, 100-200, 200-300, 300-400, 400-500, 500-591, 550-591.

It is understood that this list is exemplary only and that a fragment of this invention can be any amino acid sequence containing any combination of contiguous 15 amino acids that are numbered in the Sequence Listing as amino acids 1 through 591 even if that combination is not specifically recited as an example herein. It is also understood that these fragments can be combined in any order or amount. For example, fragment 1-10 can be combined with fragment 10-20 to produce a fragment of amino acids 1-20. Also fragments can be present in multiple numbers and in any combination 20 in a fragment of this invention. Thus, for example, fragment 1-150 can be combined with a second fragment 1-150 and/or combined with fragment 400-500 to produce a fragment of this invention. Other exemplary fragments of this invention include the domains of the CARDs toxin described herein [e.g., domain 1 (N terminal 249 amino 25 acids), domain 2 (256 amino acids) and domain 3 (247 amino acids at carboxy terminus)].

The term “homology” as used herein refers to a degree of similarity between two or more sequences. There may be partial homology or complete homology (i.e., identity). A partially complementary sequence that at least partially inhibits an identical sequence from hybridizing to a target nucleic acid is referred to as “substantially homologous.” The 30 inhibition of hybridization of the completely complementary sequence to the target

sequence can be examined using a hybridization assay (Southern or Northern blot, solution hybridization and the like) under conditions of low stringency. A substantially homologous sequence or hybridization probe will compete for and inhibit the binding of a completely homologous sequence to the target sequence under conditions of low 5 stringency, as this term is known in the art. This is not to say that conditions of low stringency are such that non-specific binding is permitted; low stringency conditions require that the binding of two sequences to one another be a specific (i.e., selective) interaction. The absence of non-specific binding can be tested by the use of a second target sequence that lacks even a partial degree of complementarity (e.g., less than about 30% 10 identity). In the absence of non-specific binding, the probe will not hybridize to the second non-complementary target sequence.

The term "hybridization" as used herein refers to any process by which a first strand of nucleic acid binds with a second strand of nucleic acid through base pairing. Nucleic acids encoding the polypeptides and/or fragments of this invention can be detected 15 by DNA-DNA or DNA-RNA hybridization or amplification using probes, primers and/or fragments of polynucleotides encoding the polypeptides and/or fragments of this invention and/or designed to detect and/or amplify the nucleic acids of this invention.

The term "hybridization complex" as used herein refers to a complex formed between two nucleic acid sequences by virtue of the formation of hydrogen bonds between 20 complementary G and C bases and between complementary A and T bases; these hydrogen bonds may be further stabilized by base stacking interactions. The two complementary nucleic acid sequences hydrogen bond in an antiparallel configuration. A hybridization complex may be formed in solution (e.g., C_0 or R_0 analysis) or between one nucleic acid sequence present in solution and another nucleic acid sequence immobilized on a solid 25 support (e.g., paper, membranes, filters, chips, pins or glass slides, or any other appropriate substrate to which cells and/or nucleic acids have been fixed).

The term "nucleotide sequence" refers to a heteropolymer of nucleotides or the sequence of these nucleotides. The terms "nucleic acid," "oligonucleotide" and "polynucleotide" are also used interchangeably herein to refer to a heteropolymer of 30 nucleotides. Generally, nucleic acid segments provided by this invention may be

assembled from fragments of the genome and short oligonucleotide linkers, or from a series of oligonucleotides, or from individual nucleotides, to provide a synthetic nucleic acid which is capable of being expressed in a recombinant transcriptional unit comprising regulatory elements derived from a microbial or viral operon, or a 5 eukaryotic gene. Nucleic acids of this invention can comprise a nucleotide sequence that can be identical in sequence to the sequence which is naturally occurring or, due to the well-characterized degeneracy of the nucleic acid code, can include alternative codons which encode the same amino acid as that which is found in the naturally occurring sequence. Furthermore, nucleic acids of this invention can comprise 10 nucleotide sequences that can include codons which represent conservative substitutions of amino acids as are well known in the art, such that the biological activity of the resulting polypeptide and/or fragment is retained.

The term "probe" or "primer" includes naturally occurring or recombinant or chemically synthesized single- and/or double-stranded nucleic acids. They can be labeled 15 for detection by nick translation, Klenow fill-in reaction, PCR or other methods well known in the art. Probes and primers of the present invention, their preparation and/or labeling are described in Sambrook et al. 1989. *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, NY and Ausubel et al. 1989. *Current Protocols in Molecular Biology*, John Wiley & Sons, New York N.Y., both of which are incorporated 20 herein by reference in their entirety for these teachings.

The term "stringent" as used here refers to hybridization conditions that are commonly understood in the art to define the conditions of the hybridization procedure. Stringency conditions can be low, high or medium, as those terms are commonly known in the art and well recognized by one of ordinary skill. In various embodiments, stringent 25 conditions can include, for example, highly stringent (i.e., high stringency) conditions (e.g., hybridization to filter-bound DNA in 0.5 M NaHPO₄, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at 65°C., and washing in 0.1xSSC/0.1% SDS at 68°C.), and/or moderately stringent (i.e., medium stringency) conditions (e.g., washing in 0.2xSSC/0.1% SDS at 42°C.).

“Amplification” as used herein includes the production of multiple copies of a nucleic acid molecule and is generally carried out using polymerase chain reaction (PCR) and/or other amplification technologies as are well known in the art (Dieffenbach and Dveksler. 1995. *PCR Primer, a Laboratory Manual*, Cold Spring Harbor Press, Plainview, N.Y.).

As used herein, the term “antibody” includes intact immunoglobulin molecules as well as fragments thereof, such as Fab, F(ab')2, and Fc, which are capable of binding the epitopic determinant of an antigen (i.e., antigenic determinant). Antibodies that bind the polypeptides of this invention are prepared using intact polypeptides or fragments containing small peptides of interest as the immunizing antigen. The polypeptide or fragment used to immunize an animal can be derived from enzymatic cleavage, recombinant expression, isolation from biological materials, synthesis, etc., and can be conjugated to a carrier protein, if desired. Commonly used carriers that are chemically coupled to peptides and proteins for the production of antibody include, but are not limited to, bovine serum albumin, thyroglobulin and keyhole limpet hemocyanin. The coupled peptide or protein is then used to immunize the animal (e.g., a mouse, rat, or rabbit). The polypeptide or peptide antigens can also be administered with an adjuvant, as described herein and as otherwise known in the art.

The term “antibody” or “antibodies” as used herein refers to all types of immunoglobulins, including IgG, IgM, IgA, IgD, and IgE. The antibody can be monoclonal or polyclonal and can be of any species of origin, including, for example, mouse, rat, rabbit, horse, goat, sheep or human, or can be a chimeric or humanized antibody. See, e.g., Walker et al., *Molec. Immunol.* 26:403-11 (1989). The antibodies can be recombinant monoclonal antibodies produced according to the methods disclosed in U.S. Patent No. 4,474,893 or U.S. Patent No. 4,816,567. The antibodies can also be chemically constructed according to the method disclosed in U.S. Patent No. 4,676,980. The antibody can further be a single chain antibody or bispecific antibody.

Antibody fragments included within the scope of the present invention include, for example, Fab, F(ab')2, and Fc fragments, and the corresponding fragments obtained

from antibodies other than IgG. Such fragments can be produced by known techniques. For example, F(ab')2 fragments can be produced by pepsin digestion of the antibody molecule, and Fab fragments can be generated by reducing the disulfide bridges of the F(ab')2 fragments. Alternatively, Fab expression libraries can be constructed to allow 5 rapid and easy identification of monoclonal Fab fragments with the desired specificity (Huse *et al.*, (1989) *Science* 254:1275-1281).

Monoclonal antibodies can be produced in a hybridoma cell line according to the technique of Kohler and Milstein, (1975) *Nature* 265:495-97. For example, a solution containing the appropriate antigen can be injected into a mouse and, after a 10 sufficient time, the mouse sacrificed and spleen cells obtained. The spleen cells are then immortalized by fusing them with myeloma cells or with lymphoma cells, typically in the presence of polyethylene glycol, to produce hybridoma cells. The hybridoma cells are then grown in a suitable medium and the supernatant screened for monoclonal antibodies having the desired specificity. Monoclonal Fab fragments can 15 be produced in bacterial cell such as *E. coli* by recombinant techniques known to those skilled in the art. See, e.g., W. Huse, (1989) *Science* 246:1275-81.

Antibodies can also be obtained by phage display techniques known in the art or by immunizing a heterologous host with a cell containing an epitope of interest.

The term "sample" as used herein is used in its broadest sense. A biological sample 20 suspected of containing a polypeptide, fragment, antibody and/or nucleic acid of this invention can be any biological fluid, an extract from a cell, an extracellular matrix isolated from a cell, a cell (in solution or bound to a solid support), a tissue, a tissue print, and the like.

"Effective amount" refers to an amount of a compound or composition of this 25 invention that is sufficient to produce a desired effect, which can be a therapeutic effect. The effective amount will vary with the age, general condition of the subject, the severity of the condition being treated, the particular agent administered, the duration of the treatment, the nature of any concurrent treatment, the pharmaceutically acceptable carrier used, and like factors within the knowledge and expertise of those skilled in the 30 art. As appropriate, an "effective amount" in any individual case can be determined by

one of ordinary skill in the art by reference to the pertinent texts and literature and/or by using routine experimentation. (See, for example, Remington, *The Science And Practice of Pharmacy* (20th ed. 2000)).

A “pharmaceutically acceptable” component such as a salt, carrier,
5 excipient or diluent of a composition according to the present invention is a component
that (i) is compatible with the other ingredients of the composition in that it can be
combined with the compositions of the present invention without rendering the
composition unsuitable for its intended purpose, and (ii) is suitable for use with
subjects as provided herein without undue adverse side effects (such as toxicity,
10 irritation, and allergic response). Side effects are “undue” when their risk outweighs
the benefit provided by the composition. Non-limiting examples of pharmaceutically
acceptable components include, without limitation, any of the standard pharmaceutical
carriers such as phosphate buffered saline solutions, water, emulsions such as oil/water
emulsion, microemulsions and various types of wetting agents.

15 “Treat,” “treating” or “treatment” refers to any type of action that imparts a
modulating effect, which, for example, can be a beneficial effect, to a subject afflicted
with a disorder, disease or illness, including improvement in the condition of the
subject (e.g., in one or more symptoms), delay in the progression of the condition,
prevention or delay of the onset of the disorder, and/or change in clinical parameters,
20 disease or illness, etc., as would be well known in the art.

A subject of this invention includes any animal susceptible to infection by *Mycoplasma pneumoniae*. Such a subject can be a mammal and in particular embodiments, is a human. A “subject in need thereof” is a subject known to be, or suspected of being, infected with *Mycoplasma pneumoniae*. A subject of this invention
25 can also include a subject not previously known or suspected to be infected by *Mycoplasma pneumoniae* or in need of treatment for *Mycoplasma pneumoniae* infection. For example, a subject of this invention can be administered the compositions of this invention even if it is not known or suspected that the subject is

infected with *Mycoplasma pneumoniae* (e.g., prophylactically). A subject of this invention is also a subject known or believed to be at risk of infection by *Mycoplasma pneumoniae*.

In certain embodiments, the fragments and/or polypeptides of this invention
5 can be fused with a “carrier” protein or peptide to produce a fusion protein. For example, the carrier protein or peptide can be fused to a polypeptide and/or fragment of this invention to increase the stability thereof (e.g., decrease the turnover rate) in the cell and/or subject. Exemplary carrier proteins include, but are not limited to, glutathione-S-transferase or maltose-binding protein. The carrier protein or peptide can
10 alternatively be a reporter protein. For example, the fusion protein can comprise a polypeptide and/or fragment of this invention and a reporter protein or peptide (e.g., Green Fluorescent Protein, β-glucuronidase, β-galactosidase, luciferase, and the like) for easy detection of transformed cells and transgene expression. As a further alternative, the fusion protein attached to the polypeptides and/or fragments and a
15 carrier protein or peptide can be targeted to a subcellular compartment of interest, i.e., to affect the co-localization of the polypeptide and/or fragment. Any suitable carrier protein as is well known in the art can be used to produce a fusion protein of this invention.

The polypeptides and/or fragments of the present invention can 1) be used
20 in assays to determine the biological activity of other proteins or peptides; 2) be included in a panel of multiple proteins for high-throughput screening; 3) be used to raise antibodies or to elicit an immune response; 4) be used as a reagent (including the labeled reagent) in assays designed to quantitatively determine levels of the protein (or its binding partner or receptor) in biological fluids; and 5) be used as markers for
25 tissues in which the corresponding protein is preferentially expressed (either constitutively or at a particular stage of tissue differentiation or development or in a disease state). Any or all of these research utilities are capable of being developed into reagent grade or kit format for commercialization as research products. Methods for

performing the uses listed above are well known to those skilled in the art. References disclosing such methods include *Molecular Cloning: A Laboratory Manual*, 2d ed., Cold Spring Harbor Laboratory Press, Sambrook et al., eds. (1989) and *Methods in Enzymology: Guide to Molecular Cloning Techniques*, Academic Press, Berger and Kimmel eds. (1987).

A variety of protocols for detecting the presence of and/or measuring the amount of polypeptides, fragments and/or peptides in a sample, using either polyclonal or monoclonal antibodies specific for the polypeptide, fragment and/or peptide are known in the art. Examples of such protocols include, but are not limited to, enzyme immunoassays (EIA), agglutination assays, immunoblots (Western blot; dot/slot blot, etc.), radioimmunoassays (RIA), immunodiffusion assays, chemiluminescence assays, antibody library screens, expression arrays, enzyme-linked immunosorbent assays (ELISA), radioimmunoassays (RIA), immunoprecipitation, Western blotting, competitive binding assays, immunofluorescence, immunohistochemical staining precipitation/flocculation assays and fluorescence-activated cell sorting (FACS). These and other assays are described, among other places, in Hampton et al. (*Serological Methods, a Laboratory Manual*, APS Press, St Paul, Minn (1990)) and Maddox et al. (*J. Exp. Med.* 158:1211-1216 (1993)).

Furthermore, a number of assays for detection and/or amplification of nucleic acid sequences are well known in the art. Additionally, a wide variety of labeling and conjugation techniques are known in the art that are used in various nucleic acid detection and amplification assays. Methods for producing labeled hybridization probes and/or PCR or other ligation primers for detecting and/or amplifying nucleic acid sequences can include, for example, oligolabeling, nick translation and end-labeling, as well as other well known methods. Alternatively, nucleic acid sequences encoding the polypeptides of this invention, and/or any functional fragment thereof, can be cloned into a plasmid or vector for detection and amplification. Such plasmids and vectors are well known in the art and are commercially available. It is also contemplated that the methods of this invention can be conducted using a variety of commercially-available

kits (e.g., Pharmacia & Upjohn; Promega; U.S. Biochemical Corp.). Suitable reporter molecules or labels, which can be used for ease of detection, include, for example, radionuclides, enzymes, fluorescence agents, chemiluminescence agents and chromogenic agents, as well as substrates, cofactors, inhibitors, magnetic particles and
5 the like as are well known in the art.

The present invention further includes isolated polypeptides, peptides, proteins, fragments, domains and/or nucleic acid molecules that are substantially equivalent to those described for this invention. As used herein, "substantially equivalent" can refer both to nucleic acid and amino acid sequences, for example a mutant sequence, that
10 varies from a reference sequence by one or more substitutions, deletions, or additions, the net effect of which does not result in an undesirable adverse functional dissimilarity between reference and subject sequences. In some embodiments, this invention can include substantially equivalent sequences that have an adverse functional dissimilarity.
15 For purposes of the present invention, sequences having equivalent biological activity and equivalent expression characteristics are considered substantially equivalent.

The invention further provides homologs, as well as methods of obtaining homologs, of the polypeptides and/or fragments of this invention from other strains of *Mycoplasma* and/or other organisms. As used herein, an amino acid sequence or protein is defined as a homolog of a polypeptide or fragment of the present invention if
20 it shares significant homology to one of the polypeptides and/or fragments of the present invention. Significant homology means at least 75%, 80%, 85%, 90%, 95%, 98% and/or 100% homology with another amino acid sequence. Specifically, by using the nucleic acids disclosed herein as a probe or as primers, and techniques such as PCR amplification and colony/plaque hybridization, one skilled in the art can identify
25 homologs of the polypeptides and/or fragments of this invention in *Mycoplasma* and/or other organisms.

The present invention also provides an antibody that specifically binds the polypeptides and/or biologically active fragments of this invention, as well as a method of making an antibody specific for a polypeptide and/or fragment of this invention
30 comprising: a) immunizing an animal with a polypeptide and/or fragment of this invention

under conditions whereby the animal produces antibodies that specifically bind the polypeptide and/or fragment of this invention; and b) removing biological materials comprising the antibodies from the animal. Also provided herein is an antibody produced by the methods set forth herein.

5 Antibodies of this invention can be generated using methods that are well known in the art. Such antibodies and immunoglobulin molecules of this invention can include, but are not limited to, polyclonal antibodies, monoclonal antibodies, chimeric antibodies, humanized antibodies, single chain antibodies (e.g., scFv), Fab fragments, and fragments produced by a Fab expression library.

10 In general, techniques for preparing polyclonal and monoclonal antibodies as well as hybridomas capable of producing a desired antibody are well known in the art. Any animal known to produce antibodies can be immunized with a polypeptide, fragment and/or antigenic epitope of this invention. Methods for immunization of animals to produce antibodies are well known in the art. For example, such methods 15 can include subcutaneous or interperitoneal injection of the polypeptide, fragment and/or antigenic epitope of this invention.

The polypeptide, fragment or antigenic epitope that is used as an immunogen can be modified or administered in an adjuvant in order to increase antigenicity. Methods of increasing the antigenicity of a protein or peptide are well known in the art 20 and include, but are not limited to, coupling the antigen with a heterologous protein (such as globulin or β -galactosidase) or through the inclusion of an adjuvant during immunization.

For example, for the production of antibodies, various hosts including goats, rabbits, rats, mice, humans, and others, can be immunized by injection with the 25 polypeptides and/or fragments of this invention, with or without a carrier protein. Additionally, various adjuvants may be used to increase the immunological response. Such adjuvants include, but are not limited to, Freund's complete and incomplete adjuvants, mineral gels such as aluminum hydroxide, and surface-active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet

hemocyanin, and dinitrophenol. Among adjuvants used in humans, BCG (bacilli Calmette-Guerin) and *Corynebacterium parvum* are especially preferable.

Polypeptides, peptides and/or fragments of this invention used as antigens to produce the antibodies of this invention can have an amino acid sequence consisting of at least five amino acids and in certain embodiments, at least ten amino acids. In one embodiment, the antigen is identical to a portion of the amino acid sequence of the natural protein, and it can contain the entire amino acid sequence of a small, naturally-occurring molecule. Short stretches of the polypeptides and/or fragments of this invention can be fused with all or a fragment of another protein that acts as a carrier protein (e.g., keyhole limpet hemocyanin) and antibodies can be produced against the chimeric polypeptide or peptide.

Monoclonal antibodies to the polypeptides and/or fragments of this invention are prepared using any technique, which provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma technique, the human B-cell hybridoma technique, and the EBV-hybridoma technique (Kohler et al. 1975. *Nature* 256:495-497; Kozbor et al. 1985. *J. Immunol. Methods* 81:31-42; Cote et al. 1983. *Proc. Natl. Acad. Sci.* 80:2026-2030; Cole et al. 1984. *Mol. Cell Biol.* 62:109-120).

For example, to produce monoclonal antibodies, spleen cells from the immunized animal are removed, fused with myeloma cells, and cultured in selective medium to become monoclonal antibody-producing hybridoma cells, according to techniques routine in the art. Any one of a number of methods well known in the art can be used to identify the hybridoma cell, which produces an antibody with the desired characteristics. These include screening the hybridomas by ELISA assay, Western blot analysis, or radioimmunoassay. Hybridomas secreting the desired antibodies are cloned and the class and subclass are identified using standard procedures known in the art.

For polyclonal antibodies, antibody-containing serum is isolated from the immunized animal and is screened for the presence of antibodies with the desired specificity using any of the well known procedures as described herein.

The present invention further provides antibodies of this invention in detectably labeled form. Antibodies can be detectably labeled through the use of radioisotopes, affinity labels (such as biotin, avidin, etc.), enzymatic labels (such as horseradish peroxidase, alkaline phosphatase, etc.) fluorescence labels (such as FITC or rhodamine, etc.), paramagnetic atoms, gold beads, etc. Such labeling procedures are well-known in the art. The labeled antibodies of the present invention can be used for *in vitro*, *in vivo*, and *in situ* assays to identify a polypeptide and/or fragment of this invention in a sample.

In some embodiments, the present invention further provides the above-described antibodies immobilized on a solid support (e.g., beads, plates, slides or wells formed from materials such as latex or polystyrene). Examples of such solid supports include plastics such as polycarbonate, complex carbohydrates such as agarose and sepharose, acrylic resins and such as polyacrylamide and latex beads. Techniques for coupling antibodies to such solid supports are well known in the art (Weir et al., 15 *Handbook of Experimental Immunology* 4th Ed., Blackwell Scientific Publications, Oxford, England, Chapter 10 (1986)). Antibodies can likewise be conjugated to detectable groups such as radiolabels (e.g., ^{35}S , ^{125}I , ^{131}I), enzyme labels (e.g., horseradish peroxidase, alkaline phosphatase), and fluorescence labels (e.g., fluorescein) in accordance with known techniques. Determination of the formation of 20 an antibody/antigen complex in the methods of this invention can be by detection of, for example, precipitation, agglutination, flocculation, radioactivity, color development or change, fluorescence, luminescence, etc., as is well known in the art.

In addition, techniques developed for the production of chimeric antibodies or humanized antibodies by splicing mouse antibody genes to human antibody genes to 25 obtain a molecule with appropriate antigen specificity and biological activity can be used (Morrison et al. 1984. *Proc. Natl. Acad. Sci.* **81**:6851-6855; Neuberger et al. 1984. *Nature* **312**:604-608; Takeda et al. 1985. *Nature* **314**:452-454). Alternatively, techniques described for the production of single chain antibodies can be adapted, using methods known in the art, to produce single chain antibodies specific for the 30 polypeptides and fragments of this invention. Antibodies with related specificity, but of

distinct idiotypic composition, can be generated by chain shuffling from random combinatorial immunoglobulin libraries (Burton 1991. *Proc. Natl. Acad. Sci.* **88**:11120-3).

Antibody fragments that specifically bind the polypeptides and/or fragments of this invention can also be generated. For example, such fragments include, but are not limited to, the F(ab')₂ fragments that can be produced by pepsin digestion of the antibody molecule and the Fab fragments that can be generated by reducing the disulfide bridges of the F(ab')₂ fragments. Alternatively, Fab expression libraries may be constructed to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity (Huse et al. 1989. *Science* **254**:1275-1281).

Various immunoassays can be used for screening to identify antibodies having the desired specificity for the proteins and peptides of this invention. Numerous protocols for competitive binding or immunoradiometric assays using either polyclonal or monoclonal antibodies with established specificity are well known in the art. Such immunoassays typically involve the measurement of complex formation between an antigen and its specific antibody (e.g., antigen/antibody complex formation). For example, a two-site, monoclonal-based immunoassay utilizing monoclonal antibodies reactive to two non-interfering epitopes on the proteins or peptides of this invention can be used, as well as a competitive binding assay.

It is further contemplated that the present invention provides kits for detection of the polypeptides and/or fragments of this invention in a sample. In one embodiment, the kit can comprise one or more antibodies of this invention, along with suitable buffers, wash solutions and/or other reagents for the detection of antibody/antigen complex formation. In an alternative embodiment, a kit of this invention can comprise a polypeptide, an antigenic peptide of the polypeptide of this invention, a fragment of this invention and/or an antigenic peptide of a fragment of this invention, along with suitable buffers, wash solutions and/or other reagents for the detection of antibody/antigen complex formation.

The present invention further provides a kit for the detection of nucleic acid encoding the polypeptides and/or fragments of this invention. For example, in one

embodiment, the kit can comprise one or more nucleic acids of this invention, along with suitable buffers, wash solutions and/or other reagents for the detection of hybridization complex formation.

It would be well understood by one of ordinary skill in the art that the kits of this
5 invention can comprise one or more containers and/or receptacles to hold the reagents (e.g., antibodies, antigens, nucleic acids) of the kit, along with appropriate buffers and/or wash solutions and directions for using the kit, as would be well known in the art. Such kits can further comprise adjuvants and/or other immunostimulatory or immunomodulating agents, as are well known in the art.

10 In further embodiments, the nucleic acids encoding the polypeptides and/or fragments of this invention can be part of a recombinant nucleic acid construct comprising any combination of restriction sites and/or functional elements as are well known in the art which facilitate molecular cloning and other recombinant DNA manipulations. Thus, the present invention further provides a recombinant nucleic acid
15 construct comprising a nucleic acid encoding a polypeptide and/or biologically active fragment of this invention.

The present invention further provides a vector comprising a nucleic acid encoding a polypeptide and/or fragment of this invention. The vector can be an expression vector which contains all of the genetic components required for expression
20 of the nucleic acid in cells into which the vector has been introduced, as are well known in the art. The expression vector can be a commercial expression vector or it can be constructed in the laboratory according to standard molecular biology protocols. The expression vector can comprise viral nucleic acid including, but not limited to, vaccinia virus, adenovirus, retrovirus and/or adeno-associated virus nucleic acid. The nucleic
25 acid or vector of this invention can also be in a liposome or a delivery vehicle, which can be taken up by a cell via receptor-mediated or other type of endocytosis.

The nucleic acid of this invention can be in a cell, which can be a cell expressing the nucleic acid whereby a polypeptide and/or biologically active fragment of this invention is produced in the cell. In addition, the vector of this invention can be
30 in a cell, which can be a cell expressing the nucleic acid of the vector whereby a

polypeptide and/or biologically active fragment of this invention is produced in the cell.

It is also contemplated that the nucleic acids and/or vectors of this invention can be present in a host animal (e.g., a transgenic animal), which expresses the nucleic acids of this invention and produces the polypeptides and/or fragments of this invention.

5 The nucleic acid encoding the polypeptide and/or fragment of this invention can be any nucleic acid that functionally encodes the polypeptides and/or fragments of this invention. To functionally encode the polypeptides and/or fragments (i.e., allow the nucleic acids to be expressed), the nucleic acid of this invention can include, for example, expression control sequences, such as an origin of replication, a promoter, an
10 enhancer and necessary information processing sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites and transcriptional terminator sequences.

Preferred expression control sequences are promoters derived from metallothionein genes, actin genes, immunoglobulin genes, CMV, SV40, adenovirus, bovine papilloma virus, etc. A nucleic acid encoding a selected polypeptide and/or
15 fragment can readily be determined based upon the genetic code for the amino acid sequence of the selected polypeptide and/or fragment and many nucleic acids will encode any selected polypeptide and/or fragment. Modifications in the nucleic acid sequence encoding the polypeptide and/or fragment are also contemplated.

Modifications that can be useful are modifications to the sequences controlling
20 expression of the polypeptide and/or fragment to make production of the polypeptide and/or fragment inducible or repressible as controlled by the appropriate inducer or repressor. Such methods are standard in the art. The nucleic acid of this invention can be generated by means standard in the art, such as by recombinant nucleic acid techniques and by synthetic nucleic acid synthesis or *in vitro* enzymatic synthesis.

25 In yet further embodiments, the present invention provides a D1 domain of CARDS Toxin comprising, consisting essentially of and/or consisting of the amino acid sequence of SEQ ID NO:69 and/or SEQ ID NO:75, a D2 domain of CARDS Toxin comprising, consisting essentially of, or consisting of the amino acid sequence of SEQ ID NO: 70, and/or a D3 domain of CARDS Toxin comprising, consisting essentially of, and/or consisting of the amino acid sequence of SEQ ID NO:71, in any combination.

Further provided herein is an isolated nucleic acid encoding the amino acid sequence of the domains D1, D2 and D3 of this invention. As one example, a nucleic acid encoding the domain D1 can comprise, consist of and/or consist essentially of the nucleotide sequence of SEQ ID NO:74.

5 Additionally provided herein are antibodies that specifically bind domain D1, D2 and/or D3 of the CARDs Toxin of this invention. The domain peptides can be used as antigens for the production of antibodies, which can be polyclonal and/or monoclonal, according to well known protocols. The domain peptides and antibodies can be used in the methods described herein for the detection of *M. pneumoniae* 10 antibodies and proteins and/or for diagnosis of *M. pneumoniae* infection, as well as in therapeutic methods to treat *M. pneumoniae* infection and related diseases as described herein.

The present invention further provides a method of producing a polypeptide and/or biologically active fragment according to the methods set forth in the Examples 15 provided herein, and as are well known in the art for polypeptide synthesis. In one embodiment, a nucleic acid encoding the polypeptides and/or fragments of this invention can be synthesized according to standard nucleic acid synthesis protocols and the nucleic acid can be expressed according to methods well known for expression of nucleic acid. The resulting polypeptide and/or fragment can then be removed from the 20 expression system by standard isolation and purification procedures and tested for any of the various biological activities described herein according to methods as taught herein as well as methods routine in the art.

The present invention also provides a method for producing the polypeptides and/or biologically active fragments of this invention comprising producing the cells of 25 this invention which contain the nucleic acids or vectors of this invention as exogenous nucleic acid; culturing the cells under conditions whereby the exogenous nucleic acid in the cell can be expressed and the encoded polypeptide and/or fragment can be produced; and isolating the polypeptide and/or fragment from the cell. Thus, it is contemplated that the polypeptides and/or fragments of this invention can be produced

in quantity *in vitro* in either prokaryotic or eukaryotic expression systems as are well known in the art.

As one example, for expression in a prokaryotic system, there are numerous *E. coli* (*Escherichia coli*) expression vectors known to one of ordinary skill in the art 5 useful for the expression of nucleic acid that encodes polypeptides. Other microbial hosts suitable for use include bacilli, such as *Bacillus subtilis*, and other enterobacteria, such as *Salmonella*, *Serratia*, as well as various *Pseudomonas* species. These prokaryotic hosts can support expression vectors that will typically contain expression control sequences compatible with the host cell (e.g., an origin of replication). In 10 addition, any number of a variety of well-known promoters can be present, such as the lactose promoter system, a tryptophan (Trp) promoter system, a beta-lactamase promoter system, or a promoter system from phage lambda. The promoters will typically control expression, optionally with an operator sequence and have ribosome binding site sequences for example, for initiating and completing transcription and 15 translation. If necessary, an amino terminal methionine can be provided by insertion of a Met codon 5' and in-frame with the polypeptide. Also, the carboxy-terminal extension of the polypeptide can be removed using standard oligonucleotide mutagenesis procedures.

The nucleic acid sequences can be expressed in hosts after the sequences have 20 been positioned to ensure the functioning of an expression control sequence. These expression vectors are typically replicable in the host organisms either as episomes or as an integral part of the host chromosomal DNA. Commonly, expression vectors can contain selection markers, e.g., tetracycline resistance or hygromycin resistance, to permit detection and/or selection of those cells transformed with the desired nucleic 25 acid sequences.

As another example, for eukaryotic system expression, a yeast expression system can be used. There are several advantages to yeast expression systems. First, evidence exists that polypeptides produced in a yeast expression system exhibit correct disulfide pairing. Second, post-translational glycosylation is efficiently carried out by 30 yeast expression systems. The *Saccharomyces cerevisiae* pre-pro-alpha-factor leader

region (encoded by the *MFa-1* gene) is routinely used to direct protein secretion from yeast. The leader region of pre-pro-alpha-factor contains a signal peptide and a pro-segment, which includes a recognition sequence for a yeast protease encoded by the *KEX2* gene. This enzyme cleaves the precursor protein on the carboxyl side of a Lys-
5 Arg dipeptide cleavage-signal sequence. The polypeptide coding sequence can be fused in-frame to the pre-pro-alpha-factor leader region. This construct is then put under the control of a strong transcription promoter, such as the alcohol dehydrogenase I promoter or a glycolytic promoter. The coding sequence is followed by a translation termination codon, which is followed by transcription termination signals.

10 Alternatively, the coding sequence of interest can be fused to a second polypeptide coding sequence, such as Sj26 or β-galactosidase, used to facilitate purification of the resulting fusion polypeptide by affinity chromatography. The insertion of protease cleavage sites to separate the components of the fusion polypeptide is applicable to constructs used for expression in yeast.

15 Efficient post-translational glycosylation and expression of recombinant polypeptides can also be achieved in *Baculovirus* systems in insect cells, as are well known in the art.

In yet further embodiments, the peptides, polypeptides and/or fragments of this invention can be expressed in mammalian cells. Mammalian cells permit the
20 expression of peptides and polypeptides in an environment that favors important post-translational modifications such as folding and cysteine pairing, addition of complex carbohydrate structures and secretion of active protein. Vectors useful for the expression of peptides and polypeptides in mammalian cells are characterized by insertion of the coding sequence between a strong (e.g., viral) promoter and a
25 polyadenylation signal. The vectors can contain genes conferring either, e.g., gentamicin or methotrexate resistance, for use as selectable markers. For example, the coding sequence can be introduced into a Chinese hamster ovary (CHO) cell line using a methotrexate resistance-encoding vector. Presence of the vector RNA in transformed cells can be confirmed by Northern blot analysis and production of a cDNA or opposite
30 strand RNA corresponding to the polypeptide or fragment coding sequence can be

confirmed by Southern and Northern blot analysis, respectively. A number of other suitable host cell lines capable of producing exogenous polypeptides have been developed in the art and include the CHO cell lines, HeLa cells, myeloma cell lines, Jurkat cells and the like. Expression vectors for these cells can include expression 5 control sequences, as described above.

The nucleic acids and/or vectors of this invention can be transferred into the host cell by well-known methods, which vary depending on the type of cell host. For example, calcium chloride transfection is commonly used for prokaryotic cells, whereas calcium phosphate treatment or electroporation can be used for other cell hosts.

10 The polypeptides, fragments, nucleic acids, vectors and cells of this invention can be present in a pharmaceutically acceptable carrier. By "pharmaceutically acceptable" is meant a material that is not biologically or otherwise undesirable, i.e., the material may be administered to an individual along with the selected polypeptide, fragment, nucleic acid, vector or cell without causing substantial deleterious biological 15 effects or interacting in a deleterious manner with any of the other components of the composition in which it is contained.

Furthermore, any of the compositions of this invention can comprise a pharmaceutically acceptable carrier and a suitable adjuvant. As used herein, "suitable adjuvant" describes an adjuvant capable of being combined with the polypeptide and/or 20 fragment and/or nucleic acid of this invention to further enhance an immune response without deleterious effect on the subject or the cell of the subject. A suitable adjuvant can be, but is not limited to, MONTANIDE ISA51 (Seppic, Inc., Fairfield, NJ), SYNTEX adjuvant formulation 1 (SAF-1), composed of 5 percent (wt/vol) squalene (DASF, Parsippany, N.J.), 2.5 percent Pluronic, L121 polymer (Aldrich Chemical, 25 Milwaukee), and 0.2 percent polysorbate (Tween 80, Sigma) in phosphate-buffered saline. Other suitable adjuvants are well known in the art and include QS-21, Freund's adjuvant (complete and incomplete), alum, aluminum phosphate, aluminum hydroxide, N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-nor-muramyl-L-alanyl-D-isoglutamine (CGP 11637, referred to as nor-MDP), N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-sn-glycero-3-

hydroxyphosphoryloxy)-ethylamine (CGP 19835A, referred to as MTP-PE) and RIBI, which contains three components extracted from bacteria, monophosphoryl lipid A, trealose dimycolate and cell wall skeleton (MPL+TDM+CWS) in 2% squalene/Tween 80 emulsion.

5 The compositions of the present invention can also include other medicinal agents, pharmaceutical agents, carriers, diluents, immunostimulatory cytokines, etc. Actual methods of preparing such dosage forms are known, or will be apparent, to those skilled in this art.

10 It is contemplated that the above-described compositions of this invention can be administered to a subject or to a cell of a subject to impart a therapeutic benefit. Thus, the present invention further provides a method of producing an immune response in a subject, comprising administering to the subject or to a cell of the subject an effective amount of a polypeptide and/or biologically active fragment of this invention and/or a nucleic acid comprising a nucleotide sequence encoding a 15 polypeptide and/or biologically active fragment of this invention. The cell of the subject can be *in vivo* or *ex vivo* and can be, but is not limited to a CD8+ T lymphocyte (e.g., a cytotoxic T lymphocyte) or an MHC I-expressing antigen presenting cell, such as a dendritic cell, a macrophage and/or a monocyte. Detection of an immune response in the subject or in the cells of the subject can be carried out according to methods 20 standard in the art for detecting a humoral and/or cellular immune response.

Furthermore, the present invention provides a method of eliciting an immune response in a subject, comprising administering to the subject an effective amount of a polypeptide and/of fragment of this invention.

Also provided herein is a method of eliciting an immune response in a subject, 25 comprising administering to the subject an effective amount of a nucleic acid and/or vector of this invention.

In additional embodiments, the present invention provides a method of providing passive immunity to a subject, comprising administering to the subject an effective amount of an antibody of this invention to the subject.

The compositions of this invention can also be employed as a therapeutic and/or prophylactic formulation and administered to a subject in need thereof. Thus, the present invention provides a method of treating or preventing infection or intoxication by *Mycoplasma pneumoniae* in a subject, comprising administering to the subject an effective amount of a polypeptide and/or fragment of this invention, a nucleic acid and/or vector of this invention, and/or an antibody of this invention.

In addition, the present invention provides a method of treating or preventing infection or intoxication caused by *Mycoplasma pneumoniae* in a subject comprising contacting an immune cell of the subject with any of the polypeptides, fragments, nucleic acids, vectors and/or antibodies of this invention. The cell can be *in vivo* or *ex vivo* and can be, for example, a CD8⁺ T cell which is contacted with the polypeptide and/or fragment of this invention in the presence of a class I MHC molecule, which can be a soluble molecule or it can be present on the surface of a cell which expresses class I MHC molecules. The cell can also be an antigen presenting cell or other class I MHC-expressing cell which can be contacted with the nucleic acids and/or vectors of this invention under conditions whereby the nucleic acid or vector is introduced into the cell by standard methods for uptake of nucleic acid and vectors. The nucleic acid encoding the polypeptide and/or fragment of this invention is then expressed and the polypeptide and/or fragment product is processed within the antigen presenting cell or other MHC I-expressing cell and presented on the cell surface as an MHC I/antigen complex. The antigen presenting cell or other class I MHC-expressing cell is then contacted with an immune cell of the subject which binds the class I MHC /antigen complex and elicits an immune response which treats or prevents *Mycoplasma pneumoniae* infection in the subject.

As set forth above, it is contemplated that in the methods wherein the compositions of this invention are administered to a subject or to a cell of a subject, such methods can further comprise the step of administering a suitable adjuvant to the subject or to a cell of the subject. The adjuvant can be in the composition of this invention or the adjuvant can be in a separate composition comprising the suitable adjuvant and a pharmaceutically acceptable carrier. The adjuvant can be administered

prior to, simultaneous with, or after administration of the composition containing any of the polypeptides, fragments, nucleic acids and/or vectors of this invention. For example, QS-21, similar to alum, complete Freund's adjuvant, SAF, etc., can be administered within days/weeks/hours (before or after) of administration of the 5 composition of this invention. The effectiveness of an adjuvant can be determined by measuring the immune response directed against the polypeptide and/or fragment of this invention with and without the adjuvant, using standard procedures, as described in the Examples herein and as are well known in the art.

As set forth above, the subject of this invention can be any subject in need of the 10 immune response of this invention and/or in need of treatment for or prevention from *Mycoplasma pneumoniae* infection, as well as any subject in whom it is desirable to induce an immune response to *Mycoplasma pneumoniae*. Symptoms of *Mycoplasma pneumoniae* infection can include tracheobronchitis and pneumonia with extrapulmonary pathologies, such as neurologic, cardiac, gastrointestinal, dermatologic, 15 renal and joint complications. A range of serological (elevated IgM and IgG seroconversion) assays and PCR detection can be used for diagnosing *M. pneumoniae* infection. Appropriate treatment can lead to resolution of respiratory symptoms such as decreased fever and cough, complete recovery of respiratory function including normal lung radiogram, and normal levels of tissue enzymes and CSF analysis. Also, 20 decreased levels of *M. pneumoniae* cells, antigens and nucleic acids in blood, sputum, bronchial lavage should accompany effective treatment.

Common sources of infection can include infected individuals coughing, sneezing and transmitting aerosols containing *M. pneumoniae*. The transmission rate is very high, which is why *M. pneumoniae* is such a common cause of community 25 acquired pneumonia. Highest targets of infection are children, especially 5-9 years old and adults between ages 25-40, although infection can occur among all 'healthy' individuals. Thus, a subject for whom the methods of this invention would be indicated for preventing *M. pneumoniae* infection can, in some embodiments, be a child or young adult.

The compositions of this invention can be administered to a cell of a subject or to a subject either *in vivo* or *ex vivo*. For administration to a cell of the subject *in vivo*, as well as for administration to the subject, the compositions of this invention can be administered orally, parenterally (e.g., intravenously), by intramuscular injection, by 5 intraperitoneal injection, subcutaneous injection, transdermally, extracorporeally, topically or the like. Also, the compositions of this invention can be pulsed onto dendritic cells, which are isolated or grown from a subject's cells, according to methods well known in the art, or onto bulk peripheral blood mononuclear cells (PBMC) or various cell subfractions thereof from a subject.

10 The exact amount of the composition required will vary from subject to subject, depending on the species, age, weight and general condition of the subject, the particular composition used, its mode of administration and the like. Thus, it is not possible to specify an exact amount for every composition of this invention. However, effective amount can be determined by one of ordinary skill in the art using only routine 15 experimentation given the teachings herein.

As an example, to a subject diagnosed with *M. pneumoniae* infection or known to be at risk of being infected with *M. pneumoniae* or in whom it is desirable to induce an immune response to *Mycoplasma pneumoniae*, between about 50-1000 nM and more preferably, between about 100-500 nM of a polypeptide and/or biologically active 20 fragment of this invention can be administered subcutaneously and can be in an adjuvant, at one to three hour/day/week intervals until an evaluation of the subject's clinical parameters indicate that the subject is not infected by *M. pneumoniae* and/or the subject demonstrates the desired immunological response. Alternatively, a polypeptide and/or fragment of this invention can be pulsed onto dendritic cells at a concentration 25 of between about 10-100 μ M and the dendritic cells can be administered to the subject intravenously at the same time intervals. The treatment can be continued or resumed if the subject's clinical parameters indicate that *M. pneumoniae* infection is present and can be maintained until the infection is no longer detected by these parameters and/or until the desired immunological response is achieved.

If *ex vivo* methods are employed, cells or tissues can be removed and maintained outside the subject's body according to standard protocols well known in the art. The polypeptides and/or biologically active fragments of this invention can be introduced into the cells via known mechanisms for uptake of polypeptides into cells (e.g., phagocytosis, pulsing onto class I MHC-expressing cells, liposomes, etc.). The cells can then be infused (e.g., in a pharmaceutically acceptable carrier) or transplanted back into the subject per standard methods for the cell or tissue type. Standard methods are known for transplantation or infusion of various cells into a subject.

The nucleic acids and vectors of this invention can also be administered to a cell of the subject either *in vivo* or *ex vivo*. The cell can be any cell that can take up and express exogenous nucleic acid and produce the polypeptides and/or fragments of this invention. In some embodiments, the polypeptides and/or fragments of this invention can be produced by a cell that secretes them, whereby the polypeptide and/or fragment is produced and secreted and then taken up and subsequently processed by an antigen presenting cell or other class I MHC-expressing cell and presented to the immune system for induction of an immune response. In other embodiments, the nucleic acids and/or vectors of this invention can be directly introduced into an antigen presenting cell and/or other class I MHC-expressing cell in which the polypeptide and/or fragment is produced and processed directly and presented to the immune system on the cell surface.

The nucleic acids and vectors of this invention can be administered orally, intranasally, parenterally (e.g., intravenously), by intramuscular injection, by intraperitoneal injection, transdermally, extracorporeally, topically or the like. In the methods described herein which include the administration and uptake of exogenous DNA into the cells of a subject (i.e., gene transduction or transfection), the nucleic acids of the present invention can be in the form of naked DNA or the nucleic acids can be in a vector for delivering the nucleic acids to the cells for expression of the polypeptides and/or fragments of this invention. The vector can be a commercially available preparation or can be constructed in the laboratory according to methods well known in the art.

Delivery of the nucleic acid or vector to cells can be via a variety of mechanisms. As one example, delivery can be via a liposome, using commercially available liposome preparations such as LIPOFECTIN, LIPOFECTAMINE (GIBCO-BRL, Inc., Gaithersburg, MD), SUPERFECT (Qiagen, Inc. Hilden, Germany) and 5 TRANSFECTAM (Promega Biotec, Inc., Madison, WI), as well as other liposomes developed according to procedures standard in the art. In addition, the nucleic acid or vector of this invention can be delivered *in vivo* by electroporation, the technology for which is available from Genetronics, Inc. (San Diego, CA) as well as by means of a SONOPORATION machine (ImaRx Pharmaceutical Corp., Tucson, AZ).

As one example, vector delivery can be via a viral system, such as a retroviral vector system, which can package a recombinant retroviral genome. The recombinant retrovirus can then be used to infect and thereby deliver to the infected cells nucleic acid encoding the polypeptide and/or fragment of this invention. The exact method of introducing the exogenous nucleic acid into mammalian cells is, of course, not limited 10 to the use of retroviral vectors. Other techniques are widely available for this procedure including the use of adenoviral vectors, alphaviral vectors, adeno-associated viral (AAV) vectors, lentiviral vectors, pseudotyped retroviral vectors and vaccinia viral vectors, as well as any other viral vectors now known or developed in the future. Physical transduction techniques can also be used, such as liposome delivery and 15 receptor-mediated and other endocytosis mechanisms. This invention can be used in conjunction with any of these or other commonly used gene transfer methods.

As another example, if the nucleic acid of this invention is delivered to the cells of a subject in an adenovirus vector, the dosage for administration of adenovirus to humans can range from about 10^7 to 10^9 plaque forming units (pfu) per injection, but 20 can be as high as 10^{12} , 10^{15} and/or 10^{20} pfu per injection. Ideally, a subject will receive a single injection. If additional injections are necessary, they can be repeated at daily/weekly/monthly intervals for an indefinite period and/or until the efficacy of the treatment has been established. As set forth herein, the efficacy of treatment can be determined by evaluating the symptoms and clinical parameters described herein and/or 25 by detecting a desired immunological response.

The exact amount of the nucleic acid or vector required will vary from subject to subject, depending on the species, age, weight and general condition of the subject, the particular nucleic acid or vector used, its mode of administration and the like. Thus, it is not possible to specify an exact amount for every nucleic acid or vector. However, 5 an appropriate amount can be determined by one of ordinary skill in the art using only routine experimentation given the teachings herein.

If *ex vivo* methods are employed, cells or tissues can be removed and maintained outside the body according to standard protocols well known in the art. The nucleic acids and vectors of this invention can be introduced into the cells via any gene 10 transfer mechanism, such as, for example, virus-mediated gene delivery, calcium phosphate mediated gene delivery, electroporation, microinjection or proteoliposomes. The transduced cells can then be infused (e.g., in a pharmaceutically acceptable carrier) or transplanted back into the subject per standard methods for the cell or tissue type. Standard methods are known for transplantation or infusion of various cells into a 15 subject.

Parenteral administration of the peptides, polypeptides, nucleic acids and/or vectors of the present invention, if used, is generally characterized by injection. Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, 20 or as emulsions. As used herein, "parenteral administration" includes intradermal, intranasal, subcutaneous, intramuscular, intraperitoneal, intravenous and intratracheal routes, as well as a slow release or sustained release system such that a constant dosage is maintained. See, e.g., U.S. Patent No. 3,610,795, which is incorporated by reference herein in its entirety.

25 The efficacy of treating or preventing *Mycoplasma pneumoniae* infection by the methods of the present invention can be determined by detecting a clinical improvement as indicated by a change in the subject's symptoms and/or clinical parameters, as would be well known to one of skill in the art.

It is further contemplated that the compositions of the present invention can be 30 used in diagnostic and therapeutic applications. Thus, the present invention provides a

method of detecting the presence of a polypeptide and/or fragment of this invention in a sample, comprising contacting the sample with an antibody of this invention under conditions whereby an antigen/antibody complex can form and detecting formation of an antigen/antibody complex, thereby detecting the presence of a *Mycoplasma pneumoniae* polypeptide and/or fragment of this invention in the sample.

5 Additionally, the present invention provides a method of detecting the presence of an antibody of this invention in a sample, comprising contacting the sample with a polypeptide and/or fragment of this invention under conditions whereby an antigen/antibody complex can form and detecting formation of an antigen/antibody complex, thereby detecting the presence of a *Mycoplasma pneumoniae* antibody of this invention in the sample.

10 The sample of this invention can be any sample in which *Mycoplasma pneumoniae* exotoxin can be present. For example, the sample can be a body fluid, cells or tissue that can contain *Mycoplasma pneumoniae* exotoxin, including but not limited to, blood, serum, plasma, saliva, sputum, bronchoalveolar lavage, urine, semen, joint fluid, cerebrospinal fluid and cells, fluids and/or tissue from all organs to which CARDS toxin can disseminate including lung, liver, heart, brain, kidney, spleen, muscle, etc.

15 Additionally, the present invention provides a method of diagnosing *Mycoplasma pneumoniae* infection in a subject comprising contacting a biological sample from the subject with a polypeptide and/or fragment of this invention under conditions whereby an antigen/antibody complex can form; and detecting formation of an antigen/antibody complex, thereby diagnosing *Mycoplasma pneumoniae* infection in the subject.

20 25 A method of diagnosing *Mycoplasma pneumoniae* infection in a subject is further provided, comprising contacting a biological sample from the subject with an antibody of this invention under conditions whereby an antigen/antibody complex can form; and detecting formation of an antigen/antibody complex, thereby diagnosing *Mycoplasma pneumoniae* infection in the subject.

30 In further embodiments, the present invention provides a method of diagnosing

infection by *Mycoplasma pneumoniae* in a subject, comprising contacting a biological sample from the subject with the nucleic acid of this invention under conditions whereby hybridization of nucleic acid molecules can occur and detecting a hybridization complex, thereby diagnosing infection by *Mycoplasma pneumoniae* in the
5 subject.

In additional embodiments, the present invention provides a method of identifying a subject infected with *Mycoplasma pneumoniae* as having a poor prognosis, comprising:

- a) establishing a correlation between the presence of and/or an amount of a
10 polypeptide, fragment, nucleic acid and/or antibody of this invention in a sample of test subjects infected with *Mycoplasma pneumoniae* and who have or had a poor prognosis;
- b) detecting in a biological sample from the subject the presence of and/or an amount of the polypeptide, fragment, nucleic acid and/or antibody of this invention correlated with a poor prognosis, thereby identifying the subject infected with
15 *Mycoplasma pneumoniae* as having a poor prognosis. For example, a correlation can be made between a level of antibodies to the CARDs toxin and a degree of respiratory and/or pulmonary dysfunction indicative of a poor prognosis.

The present invention also provides various screening assays that employ the polypeptides, fragments and/or nucleic acids of this invention. In particular, provided
20 herein is a method of identifying a substance having the ability to inhibit or enhance the binding activity of a polypeptide and/or biologically active fragment of this invention comprising contacting the substance with the CARDs protein or a biologically active fragment thereof under conditions whereby binding can occur and detecting a decrease or increase in the amount of binding in the presence of the substance as compared to a
25 control amount of binding in the absence of the substance, thereby identifying a substance having the ability to inhibit or enhance the binding activity of the CARDs toxin.

Inhibition or enhancement of binding activity can be detected by any of a variety of art-recognized methods for evaluating binding activity. As one example, the

substance to be tested and the CARDs polypeptide and/or fragment can be contacted in the presence of target cells or a target substrate (e.g., surfactant protein A; SP-A) known to bind the polypeptide or fragment. The amount of binding of polypeptide or fragment to the cells or the substrate in the presence of the substance and the amount of 5 binding of polypeptide or fragment to the cells or the substrate in the absence of the substance is determined and a decrease or increase in the amount of binding in the presence of the substance identifies the substance as having the ability to inhibit or enhance binding.

In some embodiments, binding of polypeptide and/or fragment to target cells or 10 a target substrate can be measured by attaching a detectable moiety to the polypeptide or fragment (e.g., a fluorescence moiety, histochemically detectable moiety, radioactive moiety, etc.). The amount of detectable moiety can be measured in the presence and absence of the substance to be tested and the amounts can be compared to determine inhibition or enhancement. Binding activity can also be determined by comparing the 15 amount of cytopathology observed in a monolayer of target cells in the presence and absence of the substance to be tested. Target cells that can be used in such a binding assay include, but are not limited to, Chinese hamster ovary (CHO) cells, Hep2 cells, human lung and kidney epithelial and fibroblast cells, and any other mammalian cells that exhibit sensitivity to CARDs toxin now known or later identified.

20 In addition, the present invention provides a method of identifying a substance having the ability to inhibit or enhance the translocating activity of a polypeptide and/or a biologically active fragment of this invention, comprising contacting the substance with the polypeptide of this invention and/or a biologically active fragment thereof under conditions whereby translocation activity can occur and detecting a decrease or 25 increase in the amount of translocation activity in the presence of the substance as compared to a control amount of translocation activity in the absence of the substance, thereby identifying a substance having the ability to inhibit or enhance the translocating activity of the CARDs toxin.

Inhibition or enhancement of translocating activity can be detected by any of a 30 variety of art-recognized methods for evaluating translocating activity. As one

example, the substance to be tested and the CARDs polypeptide and/or fragment can be contacted in the presence of target cells known to translocate the CARDs exotoxin. The amount of translocation of polypeptide or fragment into the cells in the presence of the substance and the amount of translocation of polypeptide or fragment into the cells
5 in the absence of the substance is determined and a decrease or increase in the amount of translocation in the presence of the substance identifies the substance as having the ability to inhibit or enhance translocation of the CARDs exotoxin. Translocation of polypeptide and/or fragment into target cells can be measured by attaching a detectable moiety to the polypeptide or fragment (e.g., a fluorescence moiety, histochemically
10 detectable moiety, radioactive moiety, etc.). The amount of translocated detectable moiety can be measured in the presence and absence of the substance to be tested and the amounts can be compared to determine inhibition or enhancement of translocation. Translocation activity can also be determined by comparing the amount of
15 cytopathology observed in a monolayer of target cells in the presence and absence of the substance to be tested. Target cells that can be used in such a translocation assay include, but are not limited to, Chinese hamster ovary (CHO) cells, etc.

Further provided is a method of identifying a substance having the ability to enhance or inhibit the immunogenic activity of the CARDs toxin of this invention and/or a biologically active fragment thereof, comprising contacting the substance with
20 the CARDs toxin or an immunogenic fragment thereof under conditions whereby a measurable immune response can be elicited and detecting an increase or decrease in the amount of immune response in the presence of the substance, as compared to a control amount of immune response in the absence of the substance, thereby identifying a substance having the ability to enhance or inhibit immunogenic activity of the
25 CARDs toxin. Assays to detect and measure immune responses are well known in the art and can be employed to detect either humoral or cellular immune responses.

In additional embodiments, the present invention provides a method of identifying a substance having the ability to inhibit or enhance the ADP-ribosylating activity of the CARDs toxin of this invention and/or biologically active fragments
30 thereof, comprising contacting the substance with the CARDs toxin or biologically

active fragment thereof under conditions whereby ADP ribosylation can occur and detecting a decrease or increase in the amount of ADP ribosylation in the presence of the substance as compared to a control amount of ADP ribosylation in the absence of the substance, thereby identifying a substance having the ability to inhibit or enhance
5 the ADP ribosylating activity of the CARDs toxin.

Methods for detecting ADP ribosylating activity are well known in the art and are described, for example, in the Examples section provided herein.

Further provided is a method of identifying a substance having the ability to inhibit or enhance the cytopathology-inducing activity of the CARDs toxin of this
10 invention and/or a biologically active fragment thereof, comprising contacting the substance with the CARDs toxin or biologically active fragment thereof under conditions whereby cytopathology (e.g., changes in cell morphology, monolayer characteristics, etc.) of target cells can be induced and detecting a decrease or increase in the amount of cytopathology in the presence of the substance, as compared to a
15 control amount of cytopathology in the absence of the substance, thereby identifying a substance having the ability to inhibit or enhance the cytopathology-inducing activity of the CARDs toxin or biologically active fragment thereof.

Methods of detecting cytopathology of cells are well known in the art and are described, for example, in the Examples section herein.

Substances identified in the screening assays of this invention to have the ability
20 to inhibit or enhance various of the activities of the polypeptides and/or fragments of this invention can be employed in methods of diagnosing *M. pneumoniae* infection, as well as in methods of treating and/or preventing *M. pneumoniae* infection. For example, such substances can be present in a pharmaceutically acceptable carrier for
25 administration to a subject and an effective amount of the substance can be administered to a subject to treat and/or prevent infection by *Mycoplasma pneumoniae*.

It is also contemplated that the present invention includes methods of screening *Mycoplasma pneumoniae* cultures for mutants defective in one or more of the

biological activities of the CARDs exotoxin, for use in a vaccine preparation. Such mutants can be identified as having a defect in any of the biological activities of the CARDs exotoxin according to the protocols described herein and as are known in the art. Such mutants can be further tested for being attenuated in the ability to produce a
5 clinical infection in a subject (i.e., for virulence potential) and then further evaluated for use as a vaccine according to known protocols.

For example, in one embodiment, CARDs toxin mutants of *Mycoplasma pneumoniae* (e.g., having a mutation in the CARDs coding sequence or lacking the CARDs coding sequence) can be generated through such art-known techniques as gene
10 disruption and their virulence potential determined by challenge studies in hamsters and by adherence and cytopathology assessments in hamster tracheal rings in organ culture and in cell culture, as is well known in the art. In addition, complementation studies can be performed to restore the defective activity of the CARDs toxin, in order to characterize the mutant.

15 The present invention is more particularly described in the following examples, which are intended as illustrative only since numerous modifications and variations therein will be apparent to those skilled in the art.

EXAMPLES

20

Mycoplasma strains and DNA isolation conditions.

M. pneumoniae reference strain M129/B9 and clinical isolates S1, L2, JL1 and RJL1 were grown to late logarithmic phase in SP-4 medium at 37°C for 72 h in 150-cm² tissue culture flasks. Mycoplasmas were harvested by washing three times with
25 PBS [150 mM NaCl, 10 mM sodium phosphate, pH 7.4] and pelleting at 12,500 g for 15 min at 4°C. *M. pneumoniae* chromosomal DNA was isolated using Easy DNA kit according to the manufacturer's protocol (Invitrogen).

30

Mycoplasma culture conditions for radiolabeling.

Wild-type *Mycoplasma pneumoniae* M129/B9 and clinical isolates were grown in SP-4 medium as above. Mycoplasma monolayers in logarithmic growth phase were washed two times with 10 ml PBS (pH 7.4) and one time with Dulbecco Modified Eagle Medium (DMEM) without L-cysteine and L-methionine and resuspended in 10 ml Dulbecco Modified Eagle Medium (DMEM) without L-cysteine and L-methionine supplemented with 10 % heat-inactivated fetal bovine serum and 100 µCi L-[³⁵S]methionine. After 4 h incubation at 37°C, supernatants were removed and monolayers washed twice with 25 ml PBS. Mycoplasma cells were scraped into a volume of 10 ml sterile PBS, collected by centrifugation at 9,500 x g and washed multiple times in PBS. Cell pellets were resuspended in 1 ml complete lysis buffer (CLB) prepared shortly before use (150 mM NaCl, 10 mM Tris, 20 µM EGTA, 0.5 M Triton-X 114, 1 mM CaCl₂ and protease inhibitors 1 µM pepstatin A, 200 µM PMSF, 1 mM N-α-p-tosyl-L-lysine chloromethyl ketone (TLCK), and 10 µM leupeptin. Cell pellets in CLB were sheared through 25 gauge needles using 3 ml syringes to obtain clear lysis. 20 µl aliquots of resuspended cell lysate were transferred to separate microfuge tubes for SDS-PAGE analysis and scintillation counter assessment (Beckman Instruments Inc. Irvine, CA). Radiolabeled lysates were diluted to 6 ml in CLB and passed through control and experimental SP-A columns (see below) in parallel.

Purification of SP-A binding proteins

A 20 x 1.2 cm control glass column was packed with 3 ml uncoupled Sepharose, another identical (experimental) column was packed with 3 ml Sepharose coupled to SP-A. Coupling of SP-A to Sepharose CL-4B was performed as follows: A total of 1.5 mg of SP-A was coupled to 2g of CNBr-activated Sepharose CL-4B according to the manufacturer's instructions except the coupling buffer was 10 mM sodium bicarbonate, pH 8.3. SP-A coupled Sepharose was stored in 5 ml of 5 mM Tris pH 7.5, containing 1mM NaN₃. Columns were equilibrated with 50 ml CLB prior to addition of radiolabeled cell lysates. Radiolabeled cell lysates were collected and reapplied to each column 3-4 times. After

samples were added, columns were washed with 10 times volume of packed material to remove unbound proteins. *M. pneumoniae* SP-A-binding proteins were eluted using a NaCl gradient (0.2 to 3 M NaCl) containing 10 mM EDTA. Eluates were collected as 1 ml fractions, and 20 µl from each fraction was assayed for specific activity with a scintillation counter.

SDS-PAGE and autoradiogram.

Fractions eluted from columns were individually dialyzed/desalting against PBS and concentrated by an Amicon concentrator/lyophilizer to 1/30th of original volume. Samples were resolved in 12% SDS-PAGE and stained with Coomassie brilliant blue or transferred to nitrocellulose and exposed to Kodak XRP-40 X-ray film (Kodak, Rochester, NY) for 4-8 days.

MALDI-TOF protein sequencing.

SDS-polyacrylamide gels containing *M. pneumoniae* SP-A binding proteins were stained with Coomassie brilliant blue and washed thoroughly in distilled water. Individual protein bands were excised from acrylamide gels and subjected to MALDI-TOF by the microsequencing facility at Baylor College of Medicine (Houston, TX).

20 Bacterial strains, plasmids and DNA manipulations.

Escherichia coli INVaF' [F'*endA1rec1hsdR17supE44gyrA96lacZM15* (*lacZYAargF*)] (Invitrogen) and *E. coli* BL21(DE3) [F'*ompT hsdS (r_B⁻ m_B⁻) gal dcm* λ(DE3) pLysS] were grown in Luria Bertani (LB) broth and used to clone and express mycoplasma CARDs toxin genes. For DNA manipulations, the following vectors were used: pCR2.1 (Ap', Km' TA cloning vector [Invitrogen]) and pET19b (Ap', N-terminal His¹⁰ tag, expression vector [Novagen]). Plasmid DNA was purified using the QIAprep spin protocol according to the manufacturer (Qiagen).

SOE-PCR

In attempting to determine precise binding motifs of *M. pneumoniae* SP-A binding

proteins, both full size and truncated overexpressed proteins are employed. Initially, the number of truncated proteins will depend upon the number and location of UGA codons. Should the possibility arise that SP-A binding motifs are located in UGA-coded regions of a protein, this issue will be addressed using full-size proteins, or protease-digested peptide 5 fragments, or synthetic peptides as described herein. UGA usage problems in genes encoding SP-A binding proteins, as well as other mycoplasma proteins, are known. In such proteins, the UGA codons in the corresponding genes are modified by site-directed mutagenesis to express full size proteins. PCR-based "splicing by overlap extension" (SOE) methods are employed to mutagenize UGA in these genes. This method is based on 10 the principle that two overlapping complementary ends may prime on each other and be extended to yield a hybrid product, and a second PCR with two primers annealing at the non-overlapping ends will amplify this hybrid. An example of a stepwise strategy for SOE-PCR is as follows. 1. 'a' and 'd' are primers for a gene and 'b' and 'c' are primers to mutagenize the UGA region. 2. Amplification carried out with primers 'a' and 'b' and 15 using genomic DNA as template gives a DNA fragment "AB" of the gene. 3. Amplification carried out with primers 'c' and 'd' and using genomic DNA as template will give DNA fragment "CD" of the gene. 4. Amplification with primers 'a' and 'd' and using DNA fragments "AB" + "CD" as templates will give the UGA modified mutant gene fragment. The overlapping primers covering the UGA codon in the genes are modified as 20 UGG, a codon that still codes for tryptophan, and the primer sets depend upon the number of UGAs to be mutated in each gene. In all cases, genomic DNA of *M. pneumoniae* is used as template, and AccuTaq polymerase mix (Sigma) is used to amplify DNA fragments.

25 Immunoblot assay

Mycoplasma total proteins or purified recombinant CARDS protein were resolved on 4-12% SDS-polyacrylamide gels (NuPAGE, InVitrogen) (His-tag released, i.e., minus His tag) and transferred electrophoretically to nitrocellulose membranes (Towbin *et al.*, 1979). Membranes were blocked for two hours with 5% (wt/vol) blotto 30 [nonfat dry milk in TBS containing 0.1% Tween-20 (TBST)], followed by three washes

with TBST, and incubated with *M. pneumoniae* infected patient sera (1:50 to 1:100 in 2% blotto) at RT for 2 h. Then, individual membranes were washed three times (15 min per wash) in TBST and incubated for 2 h (ambient temperature) with alkaline phosphatase-conjugated goat anti-human IgG Abs at a dilution of 1:2000 in TBST, 5 which were washed 5 additional times with TBST, then color developed with BCIP/NBT tablets (Sigma).

Figure 1 is an immunoblot of sera from three patients, RJ, 1970 and MJ, infected with *Mycoplasma pneumoniae*. Purified *M. pneumoniae* recombinant CARDs toxin was resolved in 4-12% SDS-PAGE and transferred to nitrocellulose membranes. 10 Membranes were blocked for two hours with 5% blotto and treated with patients' sera for two hours at room temperature. Patients' sera were diluted as follows. RJ and MJ: 1:50, and 1970: 1:100 in 2% blotto. Membranes were washed and treated with alkaline phosphatase-conjugated goat anti-human antibodies diluted 1:2000 in TBS-T and two hours and color developed. Patients RJ and MJ died within about three weeks of 15 infection and patient 1970 was hospitalized with mycoplasmal pneumonia and recovered. A 68 kDa MW recombinant CARDs toxin is indicated by the arrow; higher molecular weight and diffuse bands represent His-tagged subpopulation of recombinant CARDs toxin. Detection of antibodies to the CARDs toxin indicates *in situ* synthesis of CARDs toxin during infection and its immunogenicity.

20

Additional studies on patients infected with *M pneumoniae*.

In further studies, acute and convalescent sera were collected from patients with *Mycoplasma pneumoniae*-diagnosed respiratory infections that ranged from tracheobronchitis to bronchopneumonia. Two or three blood samples were obtained 25 from each patient. The first blood sample was collected during the acute phase of the disease, approximately two weeks following exposure to *M. pneumoniae*. The second and third "convalescent" serum samples were obtained 14 and 28 days later, respectively. Control baseline serum samples were obtained from pregnant women attending the University of Texas Health Science Center at San Antonio OB-GYN 30 clinic.

All serum samples were assessed by immunoblotting against total *M. pneumoniae* proteins. Specifically, to detect CARDs toxin protein in patients' sera, *M. pneumoniae* total cell preparations of different clinical isolated (RJ1, J1, S1 and L2) and laboratory strain (B9) were dissolved in 150 µl SDS sample buffer, boiled for two minutes and separated by SDS-PAGE using 4-12% NuPAGE SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose membranes (Schleicher & Schull, Dassel, Germany) by electroblotting. Membranes were blocked for one hour at room temperature with blocking buffer (20 mM Tris-base, 150 mM NaCl, 3% skim milk powder) and incubated with anti-CARDS Toxin mouse polyclonal antibodies diluted 1:2000 in antibody buffer (20 mM Tris-base, 150 mM NaCl, 3% skim milk powder) for one hour at 37°C. Bound IgG was detected with alkaline phosphatase (AP)-conjugated goat-antimouse IgG diluted 1:3000. Membranes were developed for 1-5 minutes with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate p-toluidinium (BCIP) solution. Results of the immunoblotting show a colored band of 68 kDa molecular weight on each membrane and thus demonstrate the presence of the CARDs toxin protein in each clinical isolate at concentrations that appear to vary among individuals.

Additional immunoblot analyses were carried out to detect antibodies to CARDs toxin in infected patients' sera wherein *M. pneumoniae* recombinant 68 kDa CARDs (rCARDs) toxin (3 µg) or the N terminal domain of CARDs toxin, rD1 (1 µg) as described herein was dissolved in 150 µl LDS sample buffer (NuPAGE), boiled for two minutes and separated by SDS-PAGE using 4-12% NuPAGE SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose membranes (Schleicher & Schull, Dassel, Germany) by electroblotting and membranes were blocked for one hour at room temperature with blocking buffer (20 mM Tris-base, 150 mM NaCl, 3% skim milk powder). Membranes were cut into 3 mm strips and incubated with human serum samples diluted 1:200 in buffer (20 mM Tris-base, 150 mM NaCl, 3% skim milk powder) for one hour at 37°C. Serum samples were from *M. pneumonia*-infected patients designated patients 1 and 2 and the first serum samples were collected during the acute phase of disease (designated 1-1 and 2-1, respectively). The second serum

samples (1-2 and 2-2) and third serum samples (1-3 and 2-3) were obtained 14 and 28 days later, respectively.

Bound IgG was detected with alkaline phosphatase (AP)-conjugated goat-antihuman IgG diluted 1:3000. Individual strips were developed for 1-5 minutes with 5 nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate p-toluidium (BCIP) solution. Results of the immunoblotting showed a colored band of 68 kDa molecular weight on each membrane containing rCARDs toxin and colored bands of 32 kDa and 28 kDa on each membrane containing the D1 domain, thus demonstrating seroconversion in these patients and detection of antibodies to the CARDs toxin, either 10 as a recombinant protein or as the D1 domain. In the latter assay, the color intensity of each band appears to increase in the samples in a manner consistent with the time course of collection from the patient during the course of the disease (i.e., 1-1<1-2<1-3) (Figure 3).

ELISAs were also carried out on the samples collected from patients 1 and 2 15 described above (i.e., samples 1-1, 1-2, 1-3, 2-1, 2-2, and 2-3). In these assays, washing at each stage was performed at least three times with PBS and sera and antibodies were diluted in 1% BSA in PBS. Each well of Immulon 4 HBX Immunoplates (Dynox) was coated overnight at 4°C with 50 µl of rCARDs toxin/D1 (1 µg/well) diluted in carbonate/bicarbonate buffer (32 mM Na₂CO₃, 64 mM NaHCO₃). Individual plates 20 were washed, 100 µl of 1 mg/ml (wt/vol) BSA in PBS was added to each well, and incubation continued for two hours at room temperature. After washing, 50 µl of diluted human serum samples (1/50 to 1/3200) were added to each well, and plates were incubated for two hours at room temperature. Then, plates were washed, and 50 µl of diluted (1:1000) alkaline phosphatase (AP)-conjugated goat-antihuman IgG 25 (Zymed) were added to each well. Plates were incubated for 1.5 hours at room temperature, washed and 50 µl of substrate solution [p-nitrophenyl phosphate (PNPP)/0.1M Tris pH 9.6] was added and plates were incubated at room temperature for 30-60 minutes. Absorbance values at 450 nm were determined for each well.

The results for patient 1 with serum dilutions of 1/100 and 1/200 and rD1 as the 30 antigen showed a decrease in optical density at the greater dilution of serum and a

stepwise increase in optical density in the samples collected sequentially during the course of disease (i.e., 1-1<1-2<1-3) (Figure 3A). This stepwise increase correlates with the increased color intensity observed with these serum samples in the immunoblot assay (Figure 3A). Similar results were obtained with sequential serum 5 samples from patient 1 when rCARDS Toxin was used as the antigen.

The results for patient 2 with serum dilutions of 1/100, 1/200, 1/400, 1/800, 1/1600 and 1/3200 and rD1 as the antigen showed a decrease in optical density as the dilution of serum increased and a stepwise increase in optical density in the samples collected sequentially during the course of disease (i.e., 2-1<2-2<2-3) (Figure 3B).

10 This stepwise increase correlates with the increased color intensity observed with these serum samples in the immunoblot assay (Figure 3B). Similar results were obtained with sequential serum samples from patient 2 when rCARDS Toxin was used as the antigen.

Additional studies were conducted wherein each well of an Immulon 4 HBX 15 Immunoplate (Dynox) was coated overnight at 4°C with 50 µl of rCARDS toxin (1, 2 or 3 µg/well) diluted in carbonate/bicarbonate buffer. After washing, 50 µl of diluted human serum samples (1/200 dilution of convalescent serum 1-3 as described above) was added to each well and plates were incubated for two hours at room temperature prior to detection of bound IgG. Negative patient serum control was also included.

20 The results showed an optical density around 1.8 and 1.9 ± SE for all three concentrations of rCARDS toxin and an optical density of the negative control around 0.6 and 0.7 ± SE for all concentrations of toxin.

A further study was carried out as described above, except that each well of Immulon 4 HBX Immunoplates (Dynox) was coated overnight at 4°C with 50 µl of 25 CARDS rD1 domain diluted as follows: 1, 2, 3, 4, 5 or 6 µg/well, in carbonate-bicarbonate buffer. Negative patient serum control was also included. The results show an optical density between 1.0 and 1.2 ± SE for all six concentrations of rD1 domain and an optical density of the negative control of 0.2 ± SE or less for all concentrations of rD1.

30 Overall, these immunoblot and ELISA studies demonstrate that both CARDS

toxin and antibodies to CARDs toxin can be detected according to the methods of this invention and that the assays can be performed with as little as 1 µg of toxin either as the recombinant protein or as the D1 domain. These studies also indicate that the D1 domain may be a better target in an ELISA format, with lower background levels.

5

Identification of *Mycoplasma pneumoniae* by PCR in sputum samples using CARDs toxin as a target DNA molecule

In this assay, phosphate buffered saline (PBS), with and without saliva, was mixed with a cell suspension (cells grown 2-3 days at 37°C in SP-4 medium; total cells 10 ~ 1 X 10⁹) of *M. pneumoniae* S1 cells in a 1:1 ratio and centrifuged. The pellet was resuspended in 200 µl of water and incubated at 4°C for 20 minutes. The sample was then boiled at 100°C for 15 minutes. 37-40 µl of this sample was used for PCR in a total reaction volume of 50 µl. The samples were serially diluted 10⁻¹ to 10⁻⁹ in PBS. PCR conditions were 95°C for five minutes; 94°C for one minute; 55°C for one minute; 15 72°C for one minute and 72°C for 10 minutes, for 30 cycles. The amplification primer set was Primer 12a forward: (nts. 1197-1220; 24 bp) 5' gcttgttctggaataccaaagagtg 3' (SEQ ID NO:23) and Primer 15a reverse: (nts. 1541-1564; 24 bp) 5' ccattctacccaatcccagctgta 3' (SEQ ID NO:26). The product size of the amplicon was 368 base pairs. Detection was by ethidium bromide staining or autoradiography with a ³²P-labeled probe. The probe used to detect the amplicon by autoradiography was Primer 20 14a forward: (nts 1371-1429; 59 bp) 5' gctggattggagggttattactataccccacaattaagtggttggtattcagatg 3' (SEQ ID NO:25). Results of this study demonstrate that *M. pneumoniae* nucleic acid can be detected in the presence or absence of saliva and that one mycoplasma cell can be identified using 25 this primer/probe set.

Cloning and sequencing of CARDs

Based on the published genome sequence of *M. pneumoniae* M129/B9 (Himmelreich *et al.*, 1996, SEQ ID NO:7), the complete open reading frame of *cards* 30 was analyzed. Translation of nucleotide sequences to amino acids revealed the

existence of eight TGA codons within the coding region of *cards*. Start and stop codons and the eight intervening TGA codons are indicated in bolded text.

```

5      ttttaattt gtaaaatttc atttttaaaa aatgccaaat cctgttagat ttgtttaccc
      tgtgatttg agaagccctg aagaaatttt tgaacatggc tttcaactt taggtgatgt
      gaaaaatttc tttgaacaca ttctctccac taatttttgtt agaagctatt ttatccac
      ttcagaaaca cccacagcag ctattcgctt ctttggtagc tggttacggg aatatgtacc
      agagcccccc agaagggtttt acttatatga aattcgtgcc gaccaacact tttacaatgc
      ccgcgcactt ggggagaact ttttagattt aatgcgtttt agacaagtag tatttgactc
      10     tggtagatcgaa gaaatggcac aaatgggaaat tagatctt cgcacttctt ttgcgtatca
      acgtgaatgg tttaccatgtt gtcctaatgc agcagctt atgcgttagt cttgactagt
      agatgctgtt cccgttgaac ctggtcatgc tcaccacccg gctggtcgtt ttgttagagac
      tactagaattt aatgaaccgg aatgcaccaa ccctcattt caagagctgc aaacccaagc
      caatgatcaa ccatgattgc caacaccagg aatagctact cctgtacattt tatcaattcc
      15     ccaagcagct tccgttgcgtt atgtttcggtt aggtacttcc gcttcgttat cgttgcgtt
      ccctgatttttga agtccacccctt ctagtaatgg tgaaaatccg ctagacaat gcattgcgg
      aaagattgtt aactataacc tacaatcctt accacagtac gctagcgtt gaaaggaact
      ggaagataca ccagtataacc taaggggaaat taaaacgcgg aaaaaccttta ttttacaagg
      agatccgcgg aataacaatgt tttttttgtt cgaagtaaac cccaaacaaa agtccagttt
      20     tccccaaacc atcttctttt gggatgttta tcaacgaatt tgcgtcaagg atttaactgg
      tgcacaaatc agtcttcgc ttactgcctt tactactcag tatgctggtc agctcaaagt
      gcaccttagt gttagcgcgg ttaatgcgtt gaacaaaag tgaaaatgtt caccgcgg
      cattgcaata actcaggtttcc gggtctcctt tgaactgtt ggtcaaaactg aaaatggctt
      gttctgaaat accaagagtgtt gtttttcaca acacgattt ttttgcgtt ctttgggggg
      25     tccaccttagt gatttggaaat aattacaaat aattgttgcgtt gatgtacta cccatgcgc
      gtttgcgttact atgcgtgcgtt ctagcacctt ctttgcgtt gttcagctt gctgggtattt
      aagggggttat tactataccc cacaattaag tggttgcgtt ttttgcgtt ctttgggggg
      tggacagata ttctatgtt taaaacttcc gaaaatcttcc ttttgcgtt ctttgggggg
      cgtttctttt ctccataata aactcaacaa acaaactggt tacagctggg atttgcgtt
      30     atgctaaaa catgacatgtt atgaggacaa agacgaaaac ttttttttttactttcgcc
      tgatgacctt accatttttcc cccgttgcgtt gcttacttcc cccacattt gctgttacgc
      tgacaaccagg cgtttaaagg tgatcataag cgggttgcgtt ttttgcgtt ggtactccac
      ttacgataaaa gtttgcgttactttaa atgtcgttgcgtt gtttttttttgcgtt gtttgcgtt
      35     ctttttagcgtt ttttgcgttactttaa acgtcactgtt gtttttttttgcgtt gtttgcgtt

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The entire *cards* sequence was amplified using forward primer 5'-
tttttacatatgcaaatcctgtt-3' (primer 1, SEQ ID NO:12) and reverse primer 5'-
gatcgcttttagcgaggatcctttaacgtt -3' (primer 2, SEQ ID NO:64), which produces *Nde*I and
*Bam*HI (underlined) sites at 5' and 3' ends of the *cards* ORF, respectively. Both
40 fragments were ligated into the pCR 2.1 vector and transformed into *E. coli* INVαF'
cells for automated sequencing using M13 forward and reverse primers.

Site-directed mutagenesis of the *cards* gene to permit expression of total
recombinant CARDS protein was necessary, which required the correction of TGAs to
TGGs in order to encode tryptophan in *E. coli*. Therefore, specific primers were
45 designed as indicated below. Primers below are also used to generate specific CARDS

domains for generating specific antibody probes.

CARDS: Oligonucleotide sequences within selected (above) nucleotide sequence.

Pri 1-16: Modified oligonucleotide sequence* to amplify the cards sequence.

5 * modified nucleotides are given in bold. Complementary oligonucleotide sequence are given underneath the reverse primers (2, 3, 5, 7, 9, 11, 13 and 15)

MPN372: 23	t ttttaaaaatgccaatccgtt	46	(SEQ ID NO:28)
10 Pri-1 : 1	t ttttacatatgccaatccgtt	24	(SEQ ID NO:12)
MPN372: 458	a atgtccgttagtgcttgact	477	(SEQ ID NO:29)
15 Pri-3 : 20	a atgtccgttagtgcttgct	1	(SEQ ID NO:30)
	ttacaggcatcacgaaccga		(SEQ ID NO:31)
MPN372: 469	t gcttgacttagatgctgtt	490	(SEQ ID NO:32)
20 Pri-4 : 1	t gcttggttagatgctgtt	22	(SEQ ID NO:33)
MPN372: 613	a tgattgccaacaccagg	630	(SEQ ID NO:34)
25 Pri-5 : 18	a tgggtgccaacaccagg	1	(SEQ ID NO:35)
	taccaacgggtgtggtcc		(SEQ ID NO:36)
MPN372: 610	a ccatgattgccaacacc	627	(SEQ ID NO:37)
30 Pri-6 : 1	a ccatgggtgccaacacc	18	(SEQ ID NO:38)
MPN372: 722	c cgtattgaagtccacctt	740	(SEQ ID NO:39)
35 Pri-7 : 19	c cgtattggagttccacctt	1	(SEQ ID NO:40)
	ggactaacctcagggtggaa		(SEQ ID NO:41)
MPN372: 717	c gtgccctgattgaagtc	734	(SEQ ID NO:42)
40 Pri-8 : 1	c gtgccctgattggagtc	18	(SEQ ID NO:43)
MPN372: 1117	a aagtggaaaaatgacaccgc	1136	(SEQ ID NO:44)
45 Pri-9 : 20	a aagtggaaaatgacaccgc	1	(SEQ ID NO:45)
	tttacaccttactgtggcg		(SEQ ID NO:46)
MPN372: 1115	c aaaagtggaaaaatgacacc	1134	(SEQ ID NO:47)
50 Pri-10: 1	c aaaagtggaaaatgacacc	20	(SEQ ID NO:48)
MPN372: 1192	a aatggcttggctgaaatacc	1213	(SEQ ID NO:49)
Pri-11: 22	a aatggcttggctgaaatacc	1	(SEQ ID NO:50)
	tttaccgaacaagacaccttatgg		(SEQ ID NO:22)
MPN372: 1197	g cttggctgaaataccaagagt	1219	(SEQ ID NO:51)

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Pri-12: 1      ||||||||| ||||||||| ||||| gcttgttctggaaatccaagagt 23      (SEQ ID NO:52)
5   MPN372: 1368 taggctggattgaagggtt 1387      (SEQ ID NO:53)
      ||||||||| ||||||| |
Pri-13: 20     taggctggattggagggtt 1      (SEQ ID NO:54)
      atccgaccataacacctccca      (SEQ ID NO:55)

10  MPN372: 1374 ggtattgaagggttattactataccccacaattaagtggttatcagatg 1429
      ||||||| ||||||||||||||||||||||||||||||||||||||| |
Pri-14: 1      ggtattggagggttattactataccccacaattaagtggttggcttatcagatg 56
      (SEQ ID NOS:56 and 57)

15  MPN372: 1541 tacagctggatttagtagaa 1561      (SEQ ID NO:58)
      ||||||| ||||||| |
Pri-15: 21     tacagctggattgggttagaa 1      (SEQ ID NO:59)
      atgtcgaccctaaccatctt      (SEQ ID NO:60)

20  MPN372: 1541 tacagctggatttagtagaa 1561      (SEQ ID NO:61)
      ||||||| ||||||| |
Pri-16: 1      tacagctggattgggttagaa 21      (SEQ ID NO:62)

25  MPN372: 1796 gatcgcttttagcgattaagctttaacg 1824      (SEQ ID NO:63)
      ||||||| ||||||| | | |||||||
Pri-2 : 28     gatcgcttttagcgaggatcctttaacg 1      (SEQ ID NO:64)
      ctagcgaaaatcgctcttagaaattgc      (SEQ ID NO:13)

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Sequence of *M. pneumoniae* CARDs.

The *cards* gene of *M. pneumoniae* reference strain M129/ B9 and clinical isolates (S1, L2, JL and RJL1) were cloned in a PCRII vector individually and sequenced.

M129/B9 represents the reference strain and S1, L2, RJL1 and JL are clinical isolates from patients in San Antonio and Dallas.

All clinical isolates have the same mutation at nucleotide 1112^(T→G) from the ATG start codon, which differs from the published reference strain. However, in clinical isolate S1 three additional nucleotide changes occur at nucleotide base positions 113^(T→C), 922^(T→C) and 1172^(T→C).

The following nucleotide changes were detected in the other clinical isolates:
 L2: 734^(A→G) and 1112^(T→G).
 40 JL: 1112^(T→G).
 RJL1: 1112^(T→G) and 1174^(T→C).

Coding sequence of S1 (*Mycoplasma pneumoniae* clinical isolate)

Bolded gs shown were introduced by site directed mutagenesis in order to express CARDs protein in *E. coli*.

5	S1 Nucleotide sequence (SEQ ID NO:8)
	atgccaatc ctgttagatt tgtttaccgt gttgatttga gaagccctga agaaaattttt 60
	gaacatggct ttcactttt aggtgatgtg agaaatttct ttgaacacat ccctccact 120
	aattttggta gaagctattt tatttccact tcagaaacac ccacagcgc tattcgcttc 180
10	tttggtagct ggtaacggga atatgtacca gaggccccca gaagggctta cttatataaa 240
	attcgtgccg accaacactt ttacaatgcc cgcccaactg gggagaactt gttagattta 300
	atgcgtcaaa gacaagtagt atttgactct ggtgatcgag aaatggcaca aatggaaatt 360
	agagctttac gcacttcctt tgcttatcaa cgtgaatggg ttaccgatgg tccaaattgca 420
15	gcagctaatttccgttagtgc ttggctagta gatgctgttc ccgttgaacc tggtcatgt 480
	caccacccgg ctggctgtgt ttagagact actagaatta atgaaccggaa aatgcacaac 540
	cctcattatc aagagctgca aacccaagcc aatgatcaac catgggtgcc aacaccaggaa 600
	atagctactc ctgtacattt atcaattccc caagcagctt ccgttgcgtga tgtttggaa 660
20	ggtaacttccg cttcgctatc gtttgcgtgc cctgatttggaa gtccacccctt tagtaatgg 720
	gaaaatccgc tagacaatg cattgcgaa aagattgata actataacctt acaatcccta 780
	ccacagtacg cttagcgtgt aaaggaaactg gaagatacac cagtataacctt aagggaaatt 840
	aaaacgcaaa aaaccccttat gttacaagca gatccgc当地 ataacaatgt cttttggc 900
	gaagtaaacc ccaaacaaaaa gcccagctt ccccaaaacca tcttcttttggatgtttat 960
	caacgaattt gtctcaagga tttaaacttggt gcacaaatca gtcttcgct tactgcctt 1020
	actactcagt atgctggtca gctcaaaatgt cacccttagtgg ttagcgc当地 taatgccgtg 1080
25	aacccaaatgt ggaaaatgac accgc当地 agac agtgc当地 ctcagttcg ggtctccctt 1140
	gaactgttag gtcaaactga aatggcttgc tecttggaaata ccaagagtggg tggttcacaa 1200
	cacgatttgt atgtatgtcc tttggaaaat ccacccatgtg atttggaaaga attacaatata 1260
	attgttgatg aatgtactac ccatgc当地 cacccttagtgg tgcgtgc当地 tagcacctt 1320
	tttggatgatg ttccatgttgc ttgttatgg aggggttatt actataaccc acaattaatgt 1380
30	gggtggctt atcagatgaa aacccaggat ggc当地 tctatgtatc aaaaacttgc 1440
	aaaatcttct ttgtccagga caacccaaaac gtgttcttcc tccataataaa actcaacaaa 1500
	caaactgggt acagctggga ttgggttagaa tggctaaaac atgacatgaa tgaggacaaa 1560
	gacaaaact ttaaatggta ctttgc当地 gatgacctt ccattccctt cgttgaaggg 1620
	cttaacttcc gccacattcg ctgttagct gacaaaccaggc agttaaaggat gatcataagc 1680
35	ggttcacgtt gggccgggtt gtactccact tacgataaaatgg tttgtatcg ttt 1740
	aagattttgg tcaaaatgtgg

Below are the amino acid sequences of individual clinical isolates

40	JL (SEQ ID NO:3)
	MPNPVRFVYR VDLRSPEEIF EHGFTSLGDV RNFFEHILST NFGRSYFIST SETPTAAIRF FGSWLREYVP EHPRRAYLYE IRADQHFYNA RATGENLLDL MRQRQVFDS GDREMAQMGI RALRTSFAYQ REWFTDGPIA AANVRSAWLV DAVPVEPGHA HHPAGRVVET TRINEPEMHN PHYQEQLTQA NDQWPWLPTPG IATPVHLSIP QAASVADVSE GTSASLSFAC PDWSPPSSNG
45	ENPLDKCIAE KIDNYNLQSL PQYASSVKEL EDTPVYLRGI KTQKTFMLQA DPQNNNVFLV EVNPKQKSSF PQTIFFWDVY QRICLKDLTG AQISLSLTAF TTQYAGQLKV HLSVSAVNAV NQWKWMTPQD SAITQFRVSS ELLGQTEENG FWNTKSGGSQ HDLYVCPLKN PPSDLEELQI IVDECTTHAQ FVTMRAASTF FVDVQLGWYW RGYYYTPQLS GWSYQMKTQD GQIFYDLKTS KIFFVQDNQN VFFLHNKLNK QTGYSWDWVE WLKHDMNEDK DENFKWYFSR DDLTIPSVEG LNFRHIRCYA DNQQLKVIIS GSRWGGWYST YDKVESNVED KILVKDGFDRL F*
50	RJL1 (SEQ ID NO:4)
	MPNPVRFVYR VDLRSPEEIF EHGFTSLGDV RNFFEHILST NFGRSYFIST SETPTAAIRF FGSWLREYVP EHPRRAYLYE IRADQHFYNA RATGENLLDL MRQRQVFDS GDREMAQMGI RALRTSFAYQ REWFTDGPIA AANVRSAWLV DAVPVEPGHA HHPAGRVVET TRINEPEMHN PHYQEQLTQA NDQWPWLPTPG IATPVHLSIP QAASVADVSE GTSASLSFAC PDWSPPSSNG
55	ENPLDKCIAE KIDNYNLQSL PQYASSVKEL EDTPVYLRGI KTQKTFMLQA DPQNNNVFLV

EVNPQKQKSSF PQTIFFWDVY QRICLKDLTG AQISLSLTAF TTQYAGQLKV HLSVASNAV
 NQWKMTMPQD SAITQFRVSS ELLGQTENGL FRNTKSGGSQ HDLYVCPLKN PPSDLEELQI
 5 IVDECTTHAQ FVTMRAASTF FVDVQLGWYW RGYYYTPQLS GWSYQMKTID QQIFYDLKTS
 KIFFVQDNQN VFFLHNKLNK QTGYSWDWVE WLKHDMNEDK DENFKWYFSR DDLTIPSVEG
 LNFRHIRCYA DNQQLKVIIS GSRWGGWYST YDKVESNVED KILVKDGFDR F*

L2 (SEQ ID NO:5)
 MPNPVRFVYR VDLRSPEEIF EHGFSTLGDV RNFFEHLST NFGRSYFIST SETPTAAIRF
 FGSQLREYVP EHPRRAYLYE IRADQHFYNA RATGENLLDL MRQRQVFDS GDREMAQMGI
 10 RALRTSFAYQ REWFTDGPIA AANVRSAWLV DAVPVEPGHA HHPAGRVVET TRINEPEMHN
 PHYQELQTQA NDQPWLPTPG IATPVHLSIP QAASVADVSE GTSASLSFAC PDWSPPSSNG
 ENPLGKCIAE KIDNYNLQSL PQYASSVKEI EDTPVYLRGI KTQKTFMLQA DPQNNNVFLV
 EVNPQKQKSSF PQTIFFWDVY QRICLKDLTG AQISLSLTAF TTQYAGQLKV HLSVASNAV
 NQWKMTMPQD SAITQFRVSS ELLGQTENGL FWNTKSGGSQ HDLYVCPLKN PPSDLEELQI
 15 IVDECTTHAQ FVTMRAASTF FVDVQLGWYW RGYYYTPQLS GWSYQMKTID QQIFYDLKTS
 KIFFVQDNQN VFFLHNKLNK QTGYSWDWVE WLKHDMNEDK DENFKWYFSR DDLTIPSVEG
 LNFRHIRCYA DNQQLKVIIS GSRWGGWYST YDKVESNVED KILVKDGFDR F*

S1 (SEQ ID NO:2)
 20 MPNPVRFVYR VDLRSPEEIF EHGFSTLGDV RNFFEHIPST NFGRSYFIST SETPTAAIRF
 FGSQLREYVP EHPRRAYLYE IRADQHFYNA RATGENLLDL MRQRQVFDS GDREMAQMGI
 RALRTSFAYQ REWFTDGPIA AANVRSAWLV DAVPVEPGHA HHPAGRVVET TRINEPEMHN
 25 PHYQELQTQA NDQPWLPTPG IATPVHLSIP QAASVADVSE GTSASLSFAC PDWSPPSSNG
 ENPLDKCIAE KIDNYNLQSL PQYASSVKEI EDTPVYLRGI KTQKTFMLQA DPQNNNVFLV
 EVNPQKQKPSF PQTIFFWDVY QRICLKDLTG AQISLSLTAF TTQYAGQLKV HLSVASNAV
 NQWKMTMPQD SAITQFRVSS ELLGQTENGL SWNTKSGGSQ HDLYVCPLKN PPSDLEELQI
 30 IVDECTTHAQ FVTMRAASTF FVDVQLGWYW RGYYYTPQLS GWSYQMKTID QQIFYDLKTS
 KIFFVQDNQN VFFLHNKLNK QTGYSWDWVE WLKHDMNEDK DENFKWYFSR DDLTIPSVEG
 LNFRHIRCYA DNQQLKVIIS GSRWGGWYST YDKVESNVED KILVKDGFDR F*

30 These sequence data are summarized below.

1. Translation of the nucleotide sequence of the clinical isolates showed changes in amino acid positions at 38, 245, 308, 371, 391 and 392.
2. All the clinical isolates have changes at amino acid position **371^{Ile→Ser}**.
- 35 3. JL had only one change at aa position **371^{Ile→Ser}**.
4. **RJL1** had one more additional change (comparing to JL) at aa position **392^{Trp→Arg}**.
5. L2 had one more additional change (comparing to JL) at aa position **245^{Asp→Gly}**.
6. S1 had three additional changes (comparing to JL) at aa positions **38^{Leu→Pro}, 308^{Ser→Pro} and 391^{Phe→Ser}**.

Expression and purification of recombinant CARDS protein.

DNA fragments were generated by digesting plasmid pCR-cards with *Nde*I and *Bam*HII and ligated into pET19b to generate pET-cards. The plasmid was transformed 45 into competent *E. coli* BL21 (DE3) cells grown to a density of 2 X 10⁹ cells/ml at 37°C

in standard LB broth containing 100 µg/ml ampicillin (Sigma-Aldrich). Induction of recombinant protein synthesis was accomplished by addition of 100 µM of isopropyl thio β-galactopyranoside (Sigma-Aldrich), and bacteria were incubated for 3 h at 37°C under aeration at 220 rpm. Cells from 1 ml samples were pelleted, resuspended in 250 µl of sample buffer (4% SDS, 125 mM Tris [pH 6.8], 10% 2-ME, 10% glycerol, 0.2% bromophenol blue), and heated to 95°C for 5 min. 10 µl aliquots of test samples were analyzed on 12% SDS/polyacrylamide gels. Recombinant colonies were screened for resistance to ampicillin and expression of a protein product of the correct size, and one recombinant clone from each construct was selected for further study. Verification of specific clones was achieved by restriction digestion and limited DNA sequencing. Fusion proteins were purified from recombinant *E. coli* under native condition by nickel affinity chromatography using the manufacturer's protocol (Qiagen).

Preparation of antisera against recombinant mycoplasma proteins.

Mice were immunized subcutaneously with 50-100 µg of recombinant total CARDs protein suspended in complete Freund's adjuvant (no peptides or truncated domains). Individual mice were boosted three times with the same amount of recombinant antigen in incomplete Freund's adjuvant at 14-day intervals. Serum samples were collected and used for immunological characterization. Monoclonal antibodies were produced using recombinant CARDs toxin and hybridoma supernatants were screened for immunoreactivity with CARDs protein and truncated peptides.

Full length recombinant CARDs Toxin (rTOX) and the amino terminal D1 domain of recombinant CARDs Toxin (rD1) were separated on 4-12% preparative gels, transferred to nitrocellulose and reacted with various concentrations (1:2, 1:10 and 1:50 or 1:100) of primary mouse antibodies against rTOX or rD1 (Monoclonal antibodies 11D1-2H10, isotype IgG1 and monoclonal antibody 19C4-2G10-1E1-2B9, isotype IgG3). Membranes were washed and reacted with alkaline phosphatase-conjugated goat anti-mouse IgG. Blots were washed again, followed by color development with NBT-BCIP reagent. Both antibodies bound a protein of

approximately 70 kDa MW in membranes containing rTOX and both antibodies bound peptides of 28 kDa MW and 32 kDa MW in membranes containing rD1.

Primers designed to express specific domains of CARDS

5 Introduced restriction sites are indicated by underline. Changes in nucleotide sequences are given in bold.

	tttttacatatgccaaatcctgtt	Primer 1	(SEQ ID NO:12)
10	tttttacatatgccaaatcctgttag	Primer 1a	(SEQ ID NO:72)
	ggatcctctacgcaatgcatttgtctag	372D1R	(SEQ ID NO:65)
15	catatgccaacaccaggaaatgctactc	372D2F	(SEQ ID NO:66)
	ggatccactaccagcctagctgaac...	372D2R	(SEQ ID NO:67)
	catatgggtcagctcaaagtgcacccctag	372D3F	(SEQ ID NO:68)
20	gatcgcttttagcgaggatccttaacg	Primer 2	(SEQ ID NO:64)

Amplified region of CARDS toxin nucleic acid encoding D1 1 (SEQ ID NO:74)

25	atgccaaatc ctgttagatt tggattaccgt gttgatttga gaagccctga agaaattttt 60
	gaacatggct tttcaacttt aggtgatgtg agaaatttct ttgaacacat tctctccact 120
	aattttggta gaagcttattt tatttccact tcagaaaacac ccacagcagc tattcgcttc 180
	tttggtagct ggtaacggga atatgtacca gagcaaaaaa gaagggctta cttatatgaa 240
	attcgtgccg accaactact ttacaatgcc cgcccaactg gggagaactt gttagattta 300
30	atgcgtcaa gacaagtagt atttgactct ggtgatcgag aaatggcaca aatgggaaatt 360
	agagctttac gcattttttc tgcttatcaa cgtgaatggt ttaccgtatgg tccaattgca 420
	gcagctaatttccatgc tggcttagta gatgtgttc ccgttgaaacc tggcatgtt 480
	caccacccgg ctggctgtgt ttttagagact actagaattt atgaacccgga aatgcacaac 540
	cctcatttttccatgc aacccaaagcc aatgtcaac catggttgcc aacaccaggaa 600
35	atacgacttc ctgtacattt atcaattccc caagcagctt ccgttgctga ttttcggaa 660
	ggtaacttccg ctgcgtatc gtttgcgtgc cctgatggta gtccacccctc tagtaatgg 720
	gaaaatccgc tagacaaatg cattgcg 747

Domains expected to be expressed in *E. coli* using the above primers.

40 Overlapping amino acids within domains are indicated by underline.

Domain 1 (SEQ ID NO:69): Primer 1 and 372D1R

1	MPNPVRFVYR VDLRSPEEIF EHGFSTLGDV RNFFEHLST NFGRSYFIST
51	SETPTAAIRF FGSWLREYVP EHPRRAYLYE IRADQHFYNA RATGENLLDL
101	MRQRQVVFDSD GDREMAQMGI RALRTSFAYQ REWFTDGPIA AANVRSAWLVL
45	DAVPVEPGHA HHPAGRVTET TRINEPEMHN PHYQELQTQA NDQP <u>PWLPTPG</u>
201	<u>IATPVHLSIP QAASVADVSE GTSASLSFAC PDWSPPSSNG ENPLDKCIA</u>

Theoretical pI/Mw: 5.54 / 28127.37

Domain 1 with His tag (underlined) (SEQ ID NO:75)

MGHHHHHHHHHHSGHIDDDDKH

1	MPNPVRFVYR VDLRSPEEIF EHGFSTLGDV RNFFEHLST NFGRSYFIST
5	SETPTAAIRF FGSLREYVP EHPRRAYLYE IRADQHFYNA RATGENLLDL
10	MRQRQVVFDS GDRREMAQMGI RALRTSFAYQ REWFTDGPIA AANVRSAWLV
15	DAVPVEPGHA HHPAGRVTET TRINEPEMHN PHYQELQTQA NDQPWLPTPG
20	<u>IATPVHLSIP QAASVADVSE GTSASLSFAC PDWSPPSSNG ENPLDKCIA</u>

Theoretical pI/Mw with the tag: 5.95 / 30894.20

10

Domain 2: (SEQ ID NO:70) 372D2F and 372D2R

PWLPTPG

20	<u>IATPVHLSIP QAASVADVSE GTSASLSFAC PDWSPPSSNG ENPLDKCIAE</u>
25	KIDNYNLQLP QPYASSVKEL EDTPVYLRLGI KTQKTFMLQA DPQNNNVFLV
30	EVNPKQKSSF PQTIFFWDVY QRICLKDLTG AQISLSLTAF TTQY <u>AGOLKV</u>
35	<u>HLSVSNAV NOKWKMTPOD IAITOFRVSS ELLGOTENGL FWNTKSGGSQ</u>
40	<u>HDLYVCPLKN PPSDLEELQI IVDECTTHAQ FVTMRAASTF FVDVQLGWY</u>

Theoretical pI/Mw: 5.05 / 28378.10

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Domain 3 (SEQ ID NO:71): 372D3F and Primer 2

AGOLKV

35	<u>HLSVSNAV NOKWKMTPOD IAITOFRVSS ELLGOTENGL FWNTKSGGSQ</u>
40	<u>HDLYVCPLKN PPSDLEELQI IVDECTTHAQ FVTMRAASTF FVDVQLGWY</u>
25	RGYYYTPQLS GWSYQMKTPD GQIFYDLKTS KIFFVQDNQN VFVFLHNKLNK
50	QTGYSWDWVE WLKHDMNEDK DENFKWYFSR DDLTIPSVEG LNFRHIRCYA
55	DNQQLKVNS GSRWGGWYST YDKVESNVED KILVKDGFDR F

Theoretical pI/Mw: 5.69 / 28966.52

30 Production of recombinant N terminal domain of CARDS Toxin rD1

To produce rD1, the D1 PCR fragment (SEQ ID NO:74) encoding the *cards* first 249 amino acids (SEQ ID NO:69) was cloned into the *E. coli* His¹⁰-tagged expression vector, pET19b (Novagen), using *Nde*1 and *Bam*HI restriction sites incorporated into the oligonucleotide primers used to amplify this nucleic acid 5' tttttacatatgccaaatcctgttag 3' (SEQ ID NO:72) and 5' ggatcc tac gcaatgcattgtctag 3' (SEQ ID NO:65). Because the *Nde*1 site in the vector overlaps an ATG start codon, cloning the D1 fragment into this site places the fragment in perfect register with the vector-derived His-tagged ribosome binding site. The amino acid sequence of the expressed protein with the His tag is shown in SEQ ID NO:75.

40 After cloning the D1 PCR fragment into pET19b and confirming the identify of the cloned fragment by DNA sequencing, a recombinant plasmid was used to transform

E. coli strain BL21 (λ DE3). Transformants were grown to mid-log phase before inducing D1 expression by addition of IPTG to a final concentration of 1 mM. After four hours, cells were harvested by centrifugation at 8000g for 15 minutes at 4°C and the pellet was resuspended in 50 mM phosphate buffer pH 8.0, containing 300 mM NaCl, 10 mM imidazole and complete, EDTA-free protease inhibitor (Sigma). Cells were disrupted by sonication; cellular debris and membranes were pelleted by centrifugation at 16000 g for 30 minutes and discarded; the supernatant was mixed with Ni-NTA agarose slurry and left on a rocker at room temperature for one hour; and then the slurry was loaded into a column. The Ni-NTA agarose packed column was 5 extensively washed with 10 mM imidazole, 20 mM imidazole, and 50 mM imidazole in the same buffer used for pellet resuspension. Finally, D1 was purified in a single step elution with 250 mM imidazole in the same buffer. Fractions containing purified protein were desalted using P10 columns (Amersham Biosciences) with TG buffer (20 mM Tris-Cl, pH 7.4, 5% glycerol) and concentrated using YM-10 (Amicon) 10 membranes. Protein concentrations were estimated using a BCA protein assay kit (Pierce) and the protein was aliquoted and stored at -80°C.

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Cytopathology in Chinese Hamster Ovary (CHO) cells

Cells were seeded into 25 cm² cell culture flasks and incubation was continued 20 until monolayer confluence was achieved. Then, recombinant CARDs protein (20 μ g/ml or 40 μ g/ml) was added for 24 hours. Monolayers were photographed on an Olympus CK40 microscope at 20X magnification.

In CHO cells, the recombinant toxin causes cytopathology with an associated "foamy" appearance, rounding of cells and cell detachment from monolayers.

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ADP-ribosylation of G proteins following incubation of CARDs protein with HEp-2 cells.

Confluent HEp-2 cells were incubated with medium alone or in the presence of 40 μ g/ml CARDs protein for 16 hours at 37°C. Cells were washed and incubated with 30 fresh medium at 37°C for four hours. Cell free extracts (CFE) were prepared and

assayed for ADP-ribosylation (CFE were incubated with 40 µg/ml CARDs protein for one hour with 0.1 µM [³²P]NAD in 100mM Tris pH 7.5, 20 mM DTT). The reaction mixture was precipitated with 10% TCA and proteins were resolved in a 4-15% gradient gel by SDS-PAGE and transferred to nitrocellulose membrane for 5 autoradiography. As shown in the autoradiogram in Figure 2, the CARDs exotoxin exhibits ADP ribosylating activity.

Animal model of *M. pneumoniae* infection

Sera from mice infected with *M. pneumoniae* has been shown to seroconvert to 10 the CARDs toxin. These mice will be used as an animal model to further assess the role of the CARDs Toxin in infection and disease progression.

Although the present process has been described with reference to specific details of certain embodiments thereof, it is not intended that such details should be regarded as limitations upon the scope of the invention except as and to the extent that 15 they are included in the accompanying claims.

Throughout this application, various patents and non-patent publications are referenced. The disclosures of these patents and publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

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